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Project Completion
Report No. 529028

BACTERIAL CONTROL OF AQUATIC ALGAL POPULATIONS - PHASE II

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United States
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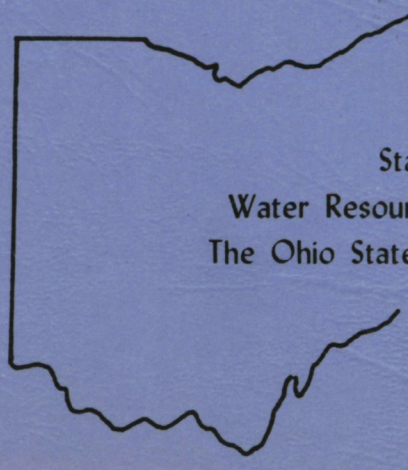

State of Ohio
Water Resources Center
The Ohio State University

A COMPLETION REPORT FOR THE GRANT ENTITLED

"Bacterial Control of Aquatic Algal Populations - Phase II"

by

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LONG RANGE GOAL

To develop an effective means of utilizing bacteria to eliminate and control bluegreen algal populations in natural water resources.

Specific Objectives of the Proposal

1 (a) To continue to determine the maximum effectiveness of the antialgal factor produced by a host-independent mutant of Bdellovibrio bacteriovorus 15143 and to examine the influence of environmental conditions on its activity.

(b) To isolate this antialgal factor and investigate the nature of its present protein dependency for synthesis.

2 (a) To test several newly isolated strains of algal inhibitory bacteria.

(b) To develop a methodology and evaluation for the concept of using these algal antagonistic bacteria to replace the natural symbiotic bacterial flora of problem-causing bluegreen algae, thereby affecting a control of the bluegreen algal population. Replacement would occur by utilization of high-growth rate antagonistic bacteria and the achievement of overgrowth.

BACKGROUND

1. General

The concept of microbiological control of pests and pollution has been recognized for many years. A principal advantage of the natural system is that introduction of non-specific toxic chemicals into the environment is avoided. Basic ecological research and succeeding field experiments have since proven the value of microorganisms in the control of unwanted populations. The use of a virus to control rabbit (Fenner, 1959) and insect (Rivers, 1964; Shea, 1971) populations has been demonstrated; and in recent years, viral control of algal populations (Safferman and Morris, 1964, 1967; Shane, 1971; Granhall, 1972; Cannon, 1974) has been partially successful

The reality of utilizing biological agents to control aquatic weeds and algae is upon us as was indicated in a recent (January, 1975) conference organized by the USEPA solely to discuss this subject. As was pointed out by Sailer (1975), in the case of weed control, 8 out of 41 projects evaluated were noted as completely successful, while 9 gave substantial control, and 14 only partial control This type of success rate clearly indicates that this concept is worth considerably more attention in the future.

This kind of approach, of artificially introducing a natural prey for an undesired host, provided the stimulus for investigating bacterial parasitism of algal population.

2. Bacteria and algal lysis

The microbial interactions between bacteria and algae that result in algal lysis include lysis of both unicellular and filamentous bluegreen algae by a Myxobacter species (Shilo, 1970). a Cytophaga species (Stewart and Brown, 1969), and the lysis of various higher algae by members of the genus Pseudomonas (Mitchell, 1972). Daft and Stewart (1971) have reported additionally on myxobacteria that lyse bluegreen algae. By plaque assay,

they demonstrated that one bacterial cell can cause algal lysis. They demonstrated the necessity of intact cells for inhibition of the algae to occur as cell filtrates had no effect on the algal cells. These authors have also reported to have had some success in limited field trials using myxobacteria to control Microcystis aeruginosa colonies (Fogg et al., 1973). Lysis in all of these cases has been caused by an exocellular enzyme that functions only using solid medium. Until lysis of Phromidium by bdellovibrios in liquid systems was reported (Burnham and Stetak, 1972), all algal lytic processes needed a solid substrate.

Vibrios and related bacteria have been shown to lyse species of algae: (a) A vibrio was reported to attach to several species of the green algal genus, Chlorella by attaching to the cells and then lysing them (Mamkaeva, 1966; Starr and Seidler, 1971) The mechanisms for this activity is not resolved. (b) Recently, a report described an antibiotic substance produced by the genus Cellvibrio, that is capable of lysing vegetative cells of the bluegreen algae, Anabaena inaequalis (Granhall and Berg, 1972). It is particularly interesting that a bacterial protease had no effect on the algicide activity nor did boiling for 15 minutes. While pepsin had no effect, papain was two-thirds inhibitory. Molecular weight determinations by filtration support the above data indicating that the weight was between 1,000 and 10,000. The activity of this substance is apparently growth-dependent as darkness inhibited its lytic effect. Another difference from the lysis caused by Myxobacter and Cytophaga is that it occurred in liquid systems and the authors suggested that the substance might play a role in the algal control in natural ecosystems (Granhall and Berg, 1972).

Coder and Starr (1978) described the algal antagonistic bacterium Bdellovibrio chlorellavorus to be effective in killing and digesting cells of the green algal genus Chlorella. These chlorellavorus bacteria attach

to the surface of the algae and penetrate the wall layers with a spike-like mechanism. The bacteria appear to be obligate symbiotes as they are unable to grow on non-living cells or on other heterotrophic nutrients.

Berland, Bonin and Maestrini (1972) looked at the toxicity of about 50 strains of bacteria for a variety of marine algae. Pseudomonas aeruginosa was found to be particularly inhibitory to Tetraselmis striata, a member of the Prasinophyceae. These authors concluded that it was not possible to state that bacteria or their byproducts are important in determining algal-bacterial relationships in the oceans.

Safferman and Morris (1962) demonstrated that actinomycete filtrates had considerable inhibitory activity against several strains of bluegreen algae. This work resulted in the suggestion that these antibiotic substances could be used as algicides. Sladekova and Sladek (1968) supported this idea of using bacterial secretion of antibiotics in the environment to control algae.

Similarly, a Bacillus brevis strain was shown to produce an extracellular product that caused lysis of both several bluegreen algal species and several bacterial species (Reim et al ,1974). This non-enzymatic substance was quite heat-stable, or low molecular weight, and could possibly be identified as an antibiotic similar to Gramicidin S. Reim et al. indicate that the utility of an anti-algal antibiotic control system may be questionable due to the inability to achieve sufficient concentrations of the inhibitor in the general environment.

3. Properties of Bdellovibrio bacteriovorus

Investigations of a unique bacterium Bdellovibrio bacteriovorus have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Shilo, 1969; Starr and Seidler, 1971). The occurrence of this organism in the natural environment is widespread

and well-documented (Shilo, 1969).

The name Bdellovibrio bacteriovorus, first proposed by Stolp and Starr (Burnham and Robinson, 1974; Stolp and Starr, 1963). adequately expresses the principal characteristics of the organisms: "Bdello" is derived from the Greek word meaning "leech"; "vibrio" denotes its shape; and "bacteriovorus" indicates that it literally eats bacteria. Scherff, DeVay and Carroll (1966) were the first to show that B. bacteriovorus attaches on gram-negative bacteria resulting in the parasite actually penetrating into the host bacterium rather than remaining on the outside. These results were supported by Starr and Baigent (1966). Lepine et al. (1967) confirmed this endoparasitism in studying a B. bacteriovorus attacking Salmonella typhi obtained from a polluted river.

Burnham, Hashimoto and Conti (1968), investigating the penetration mechanism in detail, showed that both physical and enzymatic actions combine to cause the localized breakdown of the host cell wall

The evidence to date then supports the idea that B. bacteriovorus is an endoparasite that evenually penetrates the host cell and multiplies within its confines. The cycle is completed on release of the new progeny from the host. Further refinement of the life cycle has been reported (Burnham, Hashimoto and Conti, 1970).

Antagonistic relationships between bacteria were reviewed by Stolp and Starr (1965), particularly in regard to the production of antimicrobial substances like enzymes, antiobiotics and direct microbial attacks upon another cell. The Bdellovibrio bacteriovorus system is a classical example of this latter category. The mechanisms and enzyme interactions that explain how this parasitism is successfully completed are being unraveled, but are still not totally understood (Rittenberg, 1975; Starr and Seidler, 1971). but a partial enzymatic understanding has

resulted by a recent isolation of muramidases and proteases from bdellovibrio populations (Fackrell, Campbell, Huang and Robinson, 1972; Huang and Starr, 1973). These authors point out that by itself the bdellovibrio peptidase does not lyse living cells; only heat-killed cells are susceptible to enzyme degradation. They further demonstrate that the site of activity was the mucopeptide layer of both host, Spirillum serpens, and the parasite itself (Fackrell et al., 1972).

Bacteriolysis is not solely a characteristic of the bdellovibrios as a few other groups of bacteria, notably the myxobacteria, the cytophaga and the actinomycetes are capable of lysing many bacterial strains as well as many algal species mentioned earlier. In studying the Myxobacter strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell wall lytic activity. These two functions were inseparable upon purification, making the enzyme distinct from that isolated and purified from bdellovibrios (Fackrell, Campbell, Huang and Robinson, 1972; Dr. J. Robinson, personal communication) Review of the actinomycete lysis of other microbes indicates that the responsible enzymes are peptidases (Stolp and Starr, 1965; Ghuysen, 1968), lending more support to the idea that the bdellovibrio factor or aggressin causing the breakdown of the host organism is protein in nature, and possibly a specific peptidase in activity

4. Properties of Bdellovibrio bacteriovorus lysis of bluegreen algae

Investigations of a unique parasitic bacterium Bdellovibrio bacteriovorus have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Shilo, 1969; Burnham, Hashimoto and Conti, 1968; Starr and Seidler, 1971). The occurrence of this organism in the natural environment is widespread and well-documented (Shilo, 1969).

My laboratory found that four-day old cultures of P. luridum grown in a mineral salts base could be routinely utilized has hosts for the B. bacteriovorus 15143. If equal amounts of P. luridum culture are added to a 24-hour B. bacteriovorus culture, structural alterations in the bluegreen algae could be observed continuously until the algae lysed after four days of interaction. These structural alterations included formation of refractile granules, intracellular spaces, intercellular spaces, cell swelling, breakdown of the trichomes, and finally, disintegration of the algal cell During the four-day period, the optical density of the mixed cultures gradually decreased. In all cases, the controls showed increasing cell densities throughout the week-long experiment, while the tubes with viable bdellovibrios showed total inhibition of growth and lysis of the P. luridum. Similar results can be observed for the cell-free culture supernatant of B. bacteriovorus 15143 (Burnham et al., 1976).

The bdellovibrio/P luridum interaction showed measurable chlorophyll in the system to decrease markedly and the amount of protease activity in the cultures to increase (Burnham et al.,1974; Burnham et al., 1976).

Because of these structural and pigment changes, the photosynthetic activity of the P. luridum was monitored. Over 90 percent of all O_2 production by P. luridum or M. aeruginosa was inhibited by the B. bacteriovorus whole cells or by a cell-free supernatant prepared from a 24-hour bdellovibrio culture (Sun et al..1975).

Measurement of the photosynthetic inhibition of B. bacteriovorus 15143 by the Clark electrode system showed the effect of the bdellovibrio cell-free supernatant fluids in the P. luridum. When the protein concentration was increased, the amount of inhibition of oxygen production after 1 h exposure increased beyond the 100 percent level. In other words, not only

was photosynthesis shut down by the inhibitor, but the cell respiratory rate was increased at the same time. This phenomenon was light-dependent as is indicated in the dark portion of the curve showing the respiratory rate to return to a more normal rate. Autoclaved or normal supernatant behaved similarly. Host-independent supernatant produced a similar result. It should be emphasized that control P. luridum were suspended in 4xYP for 1 h prior to being monitored for O_2 activity and did not show this phenomenon (Burnham et al., 1967; Sun et al.,1975; Burnham et al., 1977).

I have demonstrated that the bdellovibrio production of the toxin-like material is protein dependent. The higher the concentration of yeast extract present in the bdellovibrio growth medium the better the degree of photosynthetic inhibition. Control preparations containing these concentrations of protein exhibited only slight inhibition.

5. Algal lysis by members of the myxobacteriales and cytophagales

Stewart and Brown (1969) isolated a Cytophaga which formed plaques on both green and bluegreen algae. These authors indicated the lysis of the algae to be extracellular, but the exact cause of lysis was not described.

Wu et al. (1968) indicated that an unidentified myxobacterium was capable of lysing in a liquid culture strain of Lyngbya and five other bluegreen species. The authors indicated that lysis was associated with a slow "clumpy" growth of the myxobacterium and the production of a lysin.

Shilo (1970) isolated a myxobacter (designated FP-1) that lysed viable vegetative cells of many unicellular and filamentous bluegreen algae. Lysis in liquid cultures was prevented when the algal cultures were shaken. Light microscopy demonstrated that algal lysis only occurred upon polar attachment of the myxobacter to the algal cell Detection of excreted lytic enzymes was unsuccessful, suggesting that the lytic enzymes may be bound to the

surface of the myxobacter.

Five algicidal non-fruiting myxobacteria were described by Stewart and Brown (1971) to have a uniformly high G+C ratio of approximately 70 mole percent. All of these organisms were effective in lysing algae but none of these bacteria were capable of forming myxospores, a feature which distinguishes them from the Myxococcus PC02 isolate. Myxobacter has been a general name for any bacterium falling within two orders, Myxobacteriales and Cytophagales. Using the criteria described by Stewart and Brown (1971) their isolates would be grouped as members of the Cytophaga genus by the 8th edition of Bergeys Manual (Buchanan and Gibbons, 1974).

Daft and Stewart (1971) described four myxobacter that could lyse 40 strains of bluegreen algae. Again cell contact appeared to be necessary for lysis to occur. The authors suggested that one bacterium can initiate lysis of the algae. Al-hough lysis took from 2 to 7 days photosynthesis was inhibited about 85% after 10 hours. Daft and Stewart (1971) indicate that these myxobacteria may be important in regulating algal development in nature.

The structural basis for algal lysis by the Myxobacterium CP1 was described by Daft and Stewart (1973). The primary ultrastructural effect was the dissolution of the L2 or mucopeptide layer in the cell wall of the bluegreen algae tested. Large intrathylakoidal spaces were seen to form; however, the membranes themselves seemed very resistant to myxobacter CP1 disruption. This pathology of the photosynthetic system is very similar to that described for bdellovibrio interaction with Phormidium luridum (Burnham and Sun, 1977). Daft and Stewart (1973) point out that the concentration of bacteria employed in these structural studies were far in excess of those encouraged in the field. Generally, a 1:1 dilution of the bacteria with algal culture were employed.

Stewart and Daft (1977) discuss interaction within cyanobacterial blooms in fresh water Myxobacteria show a direct correlation with the number of algal cells present. The authors suggest this relationship may be due as much to excreted nutrient passing from the algae to the myxobacteria as to the use of the algae as prey by the myxobacteria.

The physiologic conditions under which algal lysis by various myxobacteria occurred was reported by Daft et al. (1975). The bacteria were all strict aerobes. Lysis increased as the pO_2 was increased to 45%. Higher levels were inhibitory. The pH optima for lysis was within the range of 7.0 to 9.0 for all strains of myxobacteria tested.

Lysis was not reported at 37 C for strain CP1. Daft et al. (1975) suggest that optimum lysis in the field should be expected in the summer months in shaoow water as the pH will also be quite suitable. These authors showed that in surveying 8 bodies of water in Scotland (5 lakes, 2 reservoirs and 1 sewage plant) there was always a direct statistical correlation between chlorophyll a concentration in the water and the abundance of these lytic bacteria. The number of myxobacteria per ml of lake water ranged from 4 to 400.

6. Myxobacterial lytic enzymes

The purpose of reviewing the following reports is to develop a list of those enzymes produced by myxobacteria that could account for the lysis of algae in clumps as reported by Burnham, Highison and Colart, 1981.

In studying the myxobacter strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell wall lytic activity. These two functions were inseparable upon purification.

Hart and Zahler (1966) studied a lysin produced by M. xanthus FBa. Purification yielded two distinct enzymes, a lysozyme and a protease. The lysozyme was very effective in lysing cell walls of various microorganisms.

Further purification of M. xanthus FB bacteriolytic enzyme was described by Sudo and Dworkin (1972). By gel separation techniques an amidase, a glucosaminidase, two proteases with amidase activity and a peptidase active against cell wall peptides were isolated. These are all individually capable of bacteriolytic activity and collectively they appear to indicate why the Myxococcus and its related genera are such potent antimicrobial parasites.

Haska (1974) purified thepeptidase produced by a related species, M. virescens and identified it to be a D-alanyl-N lysine endopeptidase, an enzyme that sould cause the destruction of the L2 (mucopeptide) layer as observed by Daft and Stewart (1973).

An alternative mechanism for the lysis of algal species could relate to the autolytic system that has been described for M. xanthus FB (Kottle and White, 1974). This enzyme system is induced during microcyst formation. The release of these enzymes which appear to result in the dissolution of cell walls could lyse walls of sensitive algal strains. Wireman and Dworkin (1977) further characterized this autolysis in terms of its sequence in the morphogenic events leading to myxospore development. The formation of the myxospore appers to be dependent on the concentration of lysis products which may provide the rationale for the autolysis.

Myxococcus xanthus strains have been shown to also produce an antibiotic active against both Gram-positive and Gram-negative bacteria. The antibiotic appears to be bacteriocidal as Escherichia coli B cells when exposed to it for 60 min showed all lysis (Rosenberg et al , 1973). Vaks et al. (1974) characterized the antibiotic to be colorless and to be active only against growing cells.

It has also been reported that many myxobacteria produce bacteriocins against sensitive bacterial strains. The host range of such substances are not known (Hirsch, 1977).

Finally it has been reported recently that proteases are bound to the extracellular slime found associated with M. virescens B2 (Gnosspelius, 1978). The author suggests that these enzymes could play an important role in denaturing protein components from microbial cells lysed by myxobacterial activities.

RESULTS

The results reported below supplement the information presented in the Appendix of the Completion Report. I am not repeating in this section the results which appear in publications resulting from this funded project.

This section describes miscellaneous results, as yet unpublished, my laboratory has obtained testing the use of microorganisms to control populations of bluegreen algae.

Part I. Previous progress reports have emphasized the interaction of the bacterial species Bdellovibrio bacteriovorus with the bluegreen algal species, P. luridum. These have shown that the bdellovibrio slowly degrade this bluegreen alga by producing a toxin-like material that interacts with the photosynthetic system, inhibiting oxygen production and causing structural lesions in the photosynthetic lamellae.

I have previously demonstrated that the bdellovibrio production of the toxin-like material (Bdellovibrio Toxin, BT) is protein-dependent. The high the concentration of yeast extract-peptone (6 mg/ml up to 15 mg/ml) present in the bdellovibrio growth medium the better the degree of photosynthetic inhibition.

I measured the number of bdellovibrio cells that were being produced in a 24 h culture when grown parasitically on E. coli with different media, i.e., 1xYP vs 4xYP. These two media will repeatedly be referred to in this report. The following is a description of their composition.

1xYP		4xYP
yeast extract (Difco)	3.0 g/l	12.0 g/l
peptone (Difco)	0.6 g/l	2.4 g/l
pH 7.2		pH 7.2

Equal amounts of E. coli were inoculated into both media. Twenty-four hours later an equal number of bdellovibrios (bd) were added to the E. coli. The results are summarized below:

Culture	#B. bacteriovorus	$\bar{n}$ by medium
Fresh Bd E. coli grown in 1xYP	1.7×10^9	2.2×10^9
4 yr old Bd E. coli grown in 1xYP	2.7×10^9	
Fresh Bd E. coli grown in 4xYP	1.6×10^8	3.3×10^9
4 yr. old Bd E. coli grown in 4xYP	6.4×10^9	

The parasitic vitality of the lyophilized strain (4 yr old) was slightly greater overall. The numbers of bdellovibrios produced by the different media was not appreciably different. The differences in photosynthetic effects on cyanobacteria due to the number of bdellovibrios present are unlikely.

The additive effect of E. coli to the inhibitory properties of the medium were rechecked. As reported in Burnham et al., 1976 the E. coli 15144 supernatant by itself in 1xYP did not significantly inhibit P. luridum photosynthesis. At 4xYP concentrations or greater, the same inhibition was observed as if the E. coli were not present.

Continued examination of the Bdellovibrio bacteriovorus interaction with the cyanobacterium Phormidium luridum included a retrospective look at the behavior of the P luridum in various media. Comparing P. luridum growth in algae broth in a mineral salts medium I found that our strains had lost approximately 80% of their photosynthetic potential in almost a linear fashion from 1974 to 1979. This pattern was found to be similar when the cyanobacteria were grown in the presence of yeast extract and peptone as well. When cyanobacterial cells were exposed to higher concentrations (12 g/l) in 1978 and 1979, I found that photosynthesis was either completely shut down or exhibited some photooxidation as I had reported previously from exposure of the cyanobacteria to extracts of B. bacteriovorus 15143.

Table 1 shows the effect of these over the various years of the study and illustrates an increasing sensitivity of this cyanobacterium to this protein source. Using 4xYP only 92% of the photosynthesis was affected in 1974; the inhibitory effect increased until 1979 so that it was only 3% of control levels. This trend was present with 1xYP. i.e., only 45% of control photosynthesis in the later years. The trend in reduced photosynthesis is also present in the control experiments, i.e., P. luridum in the mineral salts medium, "Difco Algae Broth" It appeared the cells become tired or metabolically slow due to some unknown factor The conditions of growth were constantly maintained over this period, i.e., 100 rpm shaking cultures in air exposed to 300 fc of illumination in the appropriate medium. Inocula, and the brands of media have been constant. I have checked on the batches of media and have observed no difference with media as old as two years apart so I am convinced that this effect is metabolic.

Figure 1 shows a curve of absorbance vs ul O_2/ml/hr indicating that there is a relationship between the number of algal cells present per ml and the amount of oxygen liberated during photosynthesis for P. luridum grown in AB. For cells grown in yeast extract peptone (4xYP as described earlier) the relationship is much more confused (see Fig. 2). I obtained a much greater scatter with the relationship of culture absorbance to photosynthesis much more difficult to establish. Because I have shown 4xYP to be somewhat inhibitory for P. luridum photosynthesis that is not surprising. The variability of the photosynthetic response to 4xYP has made interpretation of many of the long-term effects of algal inhibitors such as this protein or bdellovibrio-produced toxin (BT) very difficult and an unfortunate result of my research.

Near the completion of this project I tested a fresh culture of P. luridum var. olivacea obtained from the University of Texas Culture Collection. This "fresh" strain was compared to the photosynthetic bodies of the "old" strain that was used for much of my testing. The results are shown in Fig. 3. The values for corrected photosynthesis, i.e., that amount of photosynthesis produced by a culture with a density of 1.0A at 630 nm. The "fresh" P luridum produced a 3.5 fold increase in photosynthesis in AB, a 2.6 fold increase with 1xYP; and a 1.8 fold increase with 4xYP. This data was confirmed by earlier statements about a metabolically "tired" cyanobacterial culture.

In order to simplify the results we were obtaining with 4xYP I decided to eliminate the peptone from the media. The result was some decrease in ability of E. coli to grow but not a level that was significant to the experiments being conducted. Figure 4 shows that no effect on growth was observed with 1xYE (yeast extract only (3.0 g/l). Complete inhibition of growth of P. luridum was found with 4xYE (12 g/l). Correspondingly oxygen analysis showed that in AB, 1xYE and 4xYE the P. luridum cells (6-day old culture) showed a photosynthetic rate of 1.06; 0.97 and 0.00 ul O_2 produced per ml culture per hr. This serves to specify the suppressive effect the suspending medium has on the energy metabolism of the P. luridum. It also shows interpretation of algal inhibition data is difficult if yeast extract is present in the assay system.

A variety of experiments were carried out concerning the size of the photosynthetic toxin. Several of these involved the use of Amicon Ultramembrane filters with molecular cut-offs down to 500 MW. The attached table (Table 2) shows that two forms of photosynthetic inhibition may exist within the bdellovibrio supernatant. The first being contained in fractions above 1000 that show some degree of photooxidation---that is an uptake of

molecular oxygen in the presence of light. The second form simply reduces photosynthetic activity as shown by the 1000 MW and 500 MW filtrates, i.e., 94 and 89% inhibition of photosynthesis.

In measuring the protein concentration in the various filtrates of 4xYP BPT we noticed that little reduction in 280 absorption occurred.

Fraction	A	Lowry (mg/ml)
Unfiltered	1.99	3.04
30,000	1.97	2.60
10,000	1.96	2.42
5,000	1.68	1.42
1,000	1.35	1.26
500	1.20	.88

Chemical determination of protein concentration with the Lowry procedure as indicated minimized purification by molecular filtration procedures. Even though the filters are removing major proteins we are still measuring significant tyrosine and other 280 nm absorbing amino acids in the sub 1000 MW fractions. This data suggests that many free residual amino acids are present in the yeast extract-peptone-BT. This led us to test various amino acids and other small compounds for their anti-cyanobacterial effect and I will discuss these shortly.

First I will discuss that data on the photooxidative factor present in BT. Figure 5 shows the 280 nm absorbing material passing through a Sephadex (G-25) column appearing in filtrates ml 12 through 96. The inhibitory material appeared in the filtrate:ml 38-44. When compared to known proteins and dye indicators this material has a molecular weight between 4000 and 4800. Repeated column Sephadex filtration yielded the same result. This data agrees quite closely with the Amicon ultramembrane filtration data on the 5000 MW cutoff of significantly decreased photooxidation but no photosynthetic inhibition.

In extending this experimentation to much higher concentrations of yeast extrace (50 mg/ml) I found that control preparations (containing yeast extract only. but no bacterial toxins) not only shut down photosynthetic activity. but stimulate a high degree of photorespiration (i.e., -7 ul O_2/ml/hr) taken up in the light phase, compared to -0.2 ul O_2/ml/hr normally taken up in dark phase respiration. Normal healthy cells, of course, produce oxygen in the light (+1.0 ul O_2/ml/hr) Dialysis and filtration indicate the active agent in yeast extract to be small, which is much like the size of the toxin that is produced by the bdellovibrio.

This result was extremely important in that it showed that the photooxidative material was present in yeast extract and not in either the peptone component or the secretions of the bdellovibrio, i.e., the BT.

In dialysing the concentrated yeast extract (9 g/l) to further determine the size of the photooxidative component I found that neither a 14000 MW or a 3500 MW cutoff tubing allowed photorespiration. This indicates that the photooxidative component was smaller than 3500 MW.

	Exp. ½ hr	5 hr
P+AB	+.55	71
P+YE(9g/l)	-3.36	-3.50
P+YE(14,000)	+ .46	+ .30
P+YE(3,500)	+ .46	+ .57

When these dialyzed solutions were compared for protein concentration the 14000 MW dialysate possessed 5% of the original measurable protein while the 3500MW dialysate possessed 14% of the original protein. Chloroform and either extractions removed only 10% and 24% of the protein. These extractions had no effect on the photooxidation activity

We examined the comparative effects of a series of compounds suspected to mimic either the B. bacteriovorus inhibition or the inhibitory compound in yeast extract. This list included gramicidin, protamine, lysine, gelatin, casamino acids, arginine, polymyxin B, peptone, bovine serum albumin, amphotericin B, nucleic acids, cyclic adenosine monophosphate, dextrose, and various cations. These were compared in varying concentrations to fractionated preparations from the B. bacteriovorus culture as well as fractionated yeast extract preparations.

Table 3 shows the effect of substrates for yeast extract, including beside other complex proteins, series of components that are present in yeast extract in significant amounts. Also tested were dextrose, cAMP and other compounds such as each component of Davis Minimal Broth. We used Davis Minimal Broth repeatedly as a medium for the host bacterium, E. coli and wanted to be sure that no inhibitory components were present. As can be seen from the table no single component tested carried any photooxidation in the test system. Secondly only BSA of the complex proteins showed comparable inhibition to that shown by yeast extract. The mild chelating agent, sodium citrate, showed surprising inhibiting ability and should be further investigated. This was not done in my experiments.

Figure 6 shows the effect of some of these proteins and amino acids on the growth of P luridum in an AB culture. The basic amino acid lysine appeared particularly inhibitory and we proceeded to test protamine, a small highly basic protein as well. The result also shown in this figure was excellent inhibition of growth. Further experiments will be described below on the photosynthetic inhibitory ability of the compound.

The data below shows the inhibitory ability of this small basis protein, protamine, at different concentrations. Surprisingly after only one-half hr exposure a stimulatory effect was measured but this was short-lived as after 24 hr exposure to the protamine significant amounts of photosynthetic inhibition were being measured.

Exposure	Protamine Concentration	Percent Photosynthetic Inhibition	
½ hr	500 ug/ml	-10.9	stimulation
½ hr	100 ug/ml	-12.8	
½ hr	10 ug/ml	- 8.5	
½ hr	1 ug/ml	30.6	
24 hr	500 ug/ml	77.7	
24 hr	100 ug/ml	71.4	
24 hr	10 ug/ml	53.4	
24 hr	1 ug/ml	55.8	

The molecular weight separations suggested a small substance. A protein was also indicated because as shown earlier (Burnham, 1977) the production of the toxin BT was dependent upon protein concentration in the medium. Because Reim et al. (1974) had shown a small antibiotic produced by a Bacillus sp. was capable of inhibiting the cyanobacteria I decided to explore the similarities of the inhibitory effects found with BT and the photooxidative factor present in yeast extract. A comparison was to be conducted with gramicidin S and polymyxin B and that of the BT I found that 10 ug/ml gramicidin inhibited the multiplicative ability of P. luridum In addition some lysis occurred. No inhibition occurred at a concentration of 1 ug/ml. Neither did 10 ug/ml or 1 ug/ml of polymyxin B cause any significant inhibition of P. luridum growth.

The addition of 10 ug/ml Gramicidin S to the bdellovibrio supernatant caused a photooxidative effect that did not occur in controls. The data is presented below:

Test	Photosynthesis ul O_2/ml/hr	%
P + AB (1:1)	+0.65	100
P + 1xYP (1:1)	+0.65	100
P + BdS (1:1)	+0.00	0
P + BdAS (1:1)	+0.09	13.8
P + BdS + Gram (10ug/ml)	-0.42	-14.6
P + BdAS + Gram (10ug/ml)	-0.05	- 7.8

By way of comparision no effect was found by adding penicillin, bacitracin, gramicidin D or tyrothricin.

A partial explanantion for the above was discovered when I noted that almost any protein added to the gramicidin S stimulated the activity of the gramicidin on P. luridum . The following data shows this effect:

Test	Photosynthesis	%
P + AB	+1.06	100
P + 4xYP (1:1)	+0.65	61
P + 10ug/ml gramicidin (1:1)	+0.55	52
P + (4xYP + gramicidin) (1:1)	-0.14	-13

Similar effects as shown above were found when BSA 12 g/l were added to gramicidin. Following dialysis with a 3500 MW pore membrane the dialysate retained the stimulatory effect with gramicidin.

The impression that remains after exposing P. luridum to yeast extract is that a certain amount of stress on the P. luridum is found. This could account for the enhanced effects of the antibiotic. I propose this partly because 3.0 g/l yeast extract is generally stimulatory to P luridum growth while higher concentrations are inhibitory to growth and also to photosynthesis.

Scanning electron microscopy of the wall surfaces of P. luridum exposed to BdS in a 1:1 concentration show formation of lipopolysaccharide blebs 3 to 12 hours after exposure. These blebs are similar to those formed by polymyxin B on the Gram-negative bacterial lipopolysaccharides. These blebs are illustrated in Figure 7.

Another result tying this data together is the formation of blebs on the surface of the P. luridum during exposure to 45°C or 55°C for 30 min or more. When oxygen uptake as production is measured at these high temperatures I found that photooxidation was common.

I was able to determine that heating of the Phormidium luridum cultures to either 45°C or 55°C caused them to behave as if they had been exposed to the bdellovibrio toxin. Exposure at 1200 fc light of a 6-day bacteria-free P luridum culture to either a 10,000 MW fraction of a Bdellovibrio bacteriovorus15143 culture supernatant or to a 55°C environment yielded photorespiratory levels of -0.44 ul $O_2 \cdot hr^{-1} \cdot ugm$ Chl a^{-1} and -.0.30 ul $O_2 \cdot hr^{-1}$ ugm Chl a^{-1}respectively. These levels compare with 1.03 ul $O_2 \cdot hr^{-1} \cdot ugm$ Chl a^{-1} produced by control P. luridum photosynthesis at 30°C. Exposure to either heat or to the bdellovibrio supernatant produced no change from controls when the P. luridum were incubated in the dark. Phototrespiration (O_2 uptake in the light) was then approximately 4-fold higher than dark respiration (O_2 uptake in the dark). In terms of algal control experiments when the stresses (either heat treatment or bdellovibrio-produced crude toxin) were continued for longer periods of up to 24 hrs, the cells retained the ability to consume O_2 under the light even though considerable structural damage had occurred.

When the bluegreen algal culture supernatant was monitored at 260/280 nm for leakage of UV absorbing substances, it was found that during the first hour of treatment proteinaceous material predominated with leakage after 1 hour, containing significant nucleic acids.

This leakage to the environment is consistent with heat damage to bacterial membrane and to the membrane damage that occurs following bacterial membrane affecting antibiotics. The data shown by Burnham and Sun, 1977 showing extensive membrane damage to P luridum after BT exposure reinforces this.

Unfortunately and significantly I found that in high concentrations yeast extract could without the presence of P luridum cause photooxidation in the Yellow Springs oxygen analyzer. This was completely unexpected but I had never run a concentrated yeast extract alone (12 g/l or more) without P. luridum present. The data below illustrates the level of photooxidation that may occur when yeast extract is sterily introduced to the oxygen znalyzer

TEST	ul O_2/ml/hr LIGHT	DARK
P + AB (1:1)	+0.95	-0.05
P + BdAS (1:1)	+0.16	+0.09
P + YP (1:1)	+0.61	-0.23
P + YE (1:1)	+0.58	-0.21
AB + YP (1:1) (No P. luridum)	-0.09	-0.09
AB + YE (3g/l) (1:1)	-0.28	-0.12
AB + 4xYE (12 g/l) (1:1)	-1.32	-0.25
AB + 4xYP (1:1)	-0.62	-0.16
AB + peptone (1:1) (3g/l)	-0.12	-0.13

No photooxidation was observed when tryptone (3g/l), peptone (12 g/l). glucose (10 g/l), nutrient broth (3 g/l), or yeast nucleic acids (Difco) (12g/l) were used.

The table above shows that this effect is light dependent as the oxygen uptake in the dark was usually significantly less and approximately equal to the detector membrane utilization rate when incubated with water or buffer alone.

Sephadex column filtration in G-25 of the photooxidative factor confirmed it was contained in the 4000-4800 MW range with the curve exactly duplicating the curve shown in Figure 5.

This photooxidative agent in yeast extract is resistant to extraction by ether and chloroform and increases its activity up to pH 10.0. Its activity is definitely inhibited by the presence of peptone. It appears that the different responses obtained from "old" and "new" Phormidium are related to differing abilities to use proteins provided in the medium, thereby exposing the photooxidative factor and causing the different oxygen measurements.

When the BT was prepared with 1xYP or 2xYP we were not able to measure any regular photooxidative effects. These occurred when yeast extract exceeded 12 g/l. Even at that concentration occasionally and unexplanably the photooxidative effect was not present.

This photoactive agent had no effect on the growth of P luridum. The table below shows this in comparison to the inhibitory effect of a supernatant prepared from a host-independent (HI) mutant of B. bacteriovorus 15143.

	ABSORBANCE (675 nm)			
CULTURE / DAY	0	2	4	6
P + 4xYP	.33	1 73	>2.0	>2.0
P + HI BdS	.33	.41	0.22	0.17

When the bdellovibrios were grown on E. coli which were contained in dilute (1/10) yeast extract (0.3 g/l) (note the E. coli had first been grown in 4xYP, centrifuged, and then placed in the dilute medium), an excellent culture of bdellovibrio developed (3.4×10^{10}/ml). When supernatant was prepared from this no inhibition of photosynthesis occurred after one-half hour In contrast when thereplacement medium contained 1xYP or rxYP. both growth inhibition and photosynthetic inhibition occurred. This emphasizes a result reported

earlier (Burnham, 1977) that inhibitory effects of BT are protein dependent. The bdellovibrios are perhaps induced by the presence of the protein to excrete proteases and other enzymes which combine to cause cyanobacterial inhibition.

In summary. I have found that the photorespiration reported for B. bacteriovorus is due to 4000-4800 MW compound of yeasts that is present in the growth medium used for Escherichia coli, the host for B. bacteriovorus growth. When this compound is removed from the medium or from the bdellovibrio preparation, photorespiration does not occur. This compound does not by itself appear to have an inhibitory effect on the bluegreen algae P luridum. The Bdellovibrio bacteriovorus strain 15143, however, continues to surpass all other bacteria tested for production of lytic supernatants for the P luridum species.

Because of this protein dependence and because of the photooxidative complications that developed I can not recommend spending more time with this system. Instead I chose to take the information gained and apply it to working with other cyanobacterial inhibitory species.

Various other bacteria were tested for photosynthetic inhibitory ability in place of the bdellovibrios and the following results were obtained.

	ulO_2/ml culture/hr	
TEST	½ hr. exp.	4 hr exp.
P + YP (1:1)	+0.69	+1.44
P + E. coli 15143 in YP (1:1)	+0.40	+1.00
P + E. coli B/r in YP (1:1)	+0.67	+0.97
P + E. coli M107 in YP (1:1)	+0.60	+0.90
P + Klebsiella pneumoniae	+0.30	+0.40
P + Salmonella typhimurium	+0.23	+0.14
P + Staphylococcus aureus	+0.47	+0.47
P + Bacillus cereus	+0.07	+0.00

These data showed that the E. coli strains had little inhibitory activity against the P. luridum. The Salmonella typhimurium did show some promise and as the experiments described below show there is also a positive effect of the cyanobacterium on the S. typhimurium. As this organism is a pathogen I cannot recommend proceeding with utilization testing. The B. cereus as shown by Reim et al. (1974) previously secretes a small peptide antibiotic which is quite inhibitory to the cyanobacteria. Here I show that the photosynthetic system is rapidly affected.

Part II. With the disappointing results from the bdellovibrio project I began to ask questions about the general interactions that might occur between bacteria and P. luridum. If I were looking for an ideal parasite or predator of cyanobacteria perhaps it would be wise to examine the effect of cyanobacteria on bacteria, i.e., how inhibitory were cyanobacterial secretions to bacteria, or were they stimulatory. or might they have no effect. Experiments were set up using E. coli 15144, Salmonella typhimurium, and a Myxococcus species isolated from a local ditch (strain PC02).

Figure 8 shows the result of a long-term experiment in which the original culture was sequentially transferred via a 20% inoculum into fresh AB media. No heterotrophic nutrients were supplied to the survival of the bacteria--- all nutrients were either endogenous or supplied by algal metabolism or algal lysis. Figure 8a shows the control curve for P. luridum indicating a total algal population maintained at approximately 10^7 algal cells per ml The vertical lines in all the graphs indicate the numerical decrease resulting from the 20% inoculum transfer When Myxococcus PC02 was originally inoculated (1%) into a healthy P. luridum culture it can be seen in Figure 8a that this bluegreen algae could not survive and died out at the end of three weeks.

Identical algal survival curves were obtained for P. luridum plus S. typhimurium and P. luridum plus E. coli as is shown in Fig. 8a for P. luridum above. These were not illustrated because of this similarity and my desire to emphasize the effect of the bluegreen algae on bacterial survival as in Fig. 8b, c and d. If the numbers of the bacterium Myxococcus PCO2 are examined from the same experimental flask it can be seen IFig. 8b) that although there is a great deal of fluctuation, which I suspect is due to the decreasing host population of algae, the bacterial cells were able to maintain themselves at about 100 cells/ml. When Myxococcus PCO2 was placed into AB broth alone (Fig. 8b) again considerable fluctuation resulted. The bacteria demonstrate a surprising ability to withstand starvation.

I tested this bacteria/algal relationship in the same type of transfer experiment by using two other kinds of bacteria. In Fig. 8c the use of a pathogen Salmonella typhimurium was tested. As can be seen, the bacterium was not able to survive in the autotrophic medium alone and died out over 17 days. Significantly the algae appeared to offer a protective viability to the bacterium by preventing its death or extinction via dilution over 32 days. The significance of a competing algal antagonistic bacterial population such as that offered by Myxococcus PCO2 becomes enhanced here as it appears possible that some algae can aid in the survival of pathogenic bacteria in an autotrophic aqueous environment. This was recently demonstrated for the survival of the Legionalla pneumophilia bacterium, the etiologic agent of Legionaire's Disease (Tison et al.,1980).

When Escherichia coli was tested under similar conditions the trend at the end of this experiment suggested that the algae again were slightly aiding in the survival of the bacteria. Other experiments have shown a much faster decline in viability of the AB suspended E. coli so additional data is being collected to clarify this bacterium's response to no heterotrophic nutrient.

This work is being presently done by Connie Basch, a senior at Maumee Valley Country Day School working in my laboratory.

Some very exciting results can be reported involving the discovery of the predatory nature of the myxococci in agitated aqueous systems. As discussed in the Background this group is well-known for its antibacterial and anti-cyanobacterial properties but the colonial predatory ability of this group had not been described. The results below provide some data on this system which is extensively explained inthe manuscripts (Burnham et al., 1981; 1982) present in the appendix.

During a sampling trip in May. 1978 a sample was obtained from a roadside ditch on Route 2 in Port Clinton Ohio. This sample when struck out on bluegreen algal lawn of P luridum showed excellent algal lysis that spread over several days to the edges of the petri plate. The lytic regions were picked and struck on several media with the dilute medium containing 0.2% tryptone showing bacterial colonies of an orange colored spreading colony with fruiting structures forming at concentric intervals from the inoculum.

The organism was tentatively identified as a member of the Myxobacteria (McCurdy, 1974) and given the strain designation Myxococcus PC02 for its site of origin (Port Clinton, Ohio, Route 2)

Physiological tests were carried out as follows by utilizing the API 20E test series (Analytical Products, N.Y.):

1.	ONPG hydrolysis of B-galactosidase	Negative
2.	Arginine dihydrolase	Negative
3.	Lysine decarboxylase	Negative
4.	Ornithine decarboxylase	Negative
5.	Citrate utilization	Negative
6.	Hydrogen sulfide production	Negative
7.	Urease	Negative
8.	Tryptophase deaminase	Negative
9.	Indole formation	Negative
10.	Acetoin production from sodium pyruvate	Negative
11.	Gelatin liquefacation	Positive
12.	Glucose utilization	Negative
13.	Mannose utilization	Negative
14.	Inositol utilization	Negative
15.	Sorbitol utilization	Negative
16.	Rhamnose utilization	Negative
17.	Sucrose utilization	Negative
18.	Melibiose utilization	Negative
19.	Amygdalin utilization	Negative
20.	Arabinose utilization	Negative
21.	Catalase production	Positive
22.	Cellulase production	Negative

The bacteria are not flagellated but show a slow gliding motility typical of Myxococcaceae. Shaking liquid cultures snow yellowish-orange balls and clumps of Myxococcus PC02. The individual cells of the bacterium are rods with a diameter of 0.8 um and a length of between 4 and 7 um. If an agar culture is allowed to sit in the laboratory over a week refractile muxospores form on the older colonies. The diameter of these ovoid structures is 2 um. The refractile nature of these microcysts is lost upon their germination into vegetative cells. The size of the microcyst is important in the classification of the Myxococcus species and rules out M. fulvus, M. stipitatus and M. coraloides, but leaves the possibilities of M. xanthus, M. virescens and M. macrosporus. From the fruiting structures it appears that the best choice is a strain of M. xanthus (McCurdy, 1974), but further work needs to be done.

The antibiotic sensitivity pattern with the width (radius from disc) of the zones of inhibition noted is as follows:

1.	sulfamethoxyzole trimethoprim, 23.7 ugm/1.25 ugm	S*	25mm
2.	erythromycin, 15 ugm	S	10mm
3.	neomycin, 5 ugm	R**	1mm
4.	streptomycin, 10 ugm	S	12mm
5.	gentamicin, 10 ugm	R	1mm
6.	bacitracin, 10 units	R	1mm
7.	tetracycline, 5 ugm	S	17mm
8.	kanamycin, 5 ugm	S	12mm
9.	ampicillin, 10 ugm	R	0mm
10.	penicillin, 10 units	R	0mm
11.	polymyxin B, 50 units	S	5mm
12.	actinomycin D, 5 ugm	S	10mm
13.	chloramphenicol, 50 ugm	R	0mm

* sensitive ** resistant

This test was carried out using BBL (Becton, Dickinson and Co.,Cockeysville, Md.). Sensidiscs placed on 0.2% tryptone medium inoculated by spreading Myxococcus PC02 over the agar surface. The pattern is distinct from that reported by Stewart and Brown (1971) in that penicillin resistance is so marked. McCurdy (1974) reported M. xanthus to be resistant to 5 ugm streptomycin discs. The PC02 strain is sensitive to 10 ugm streptomycin so further testing is needed.

When Myxococcus PC02 is grown in 0.2% tryptone in shaking flasks at 30°C maximum growth was achieved in three daysl Viability remained at approximately 10^6/ml when tested for 16 days which indicated a reasonable ability of these bacteria to survive under less than optimum conditions. The curve also indicates that the early autolysis rate did not continue.

In direct contrast to the results reported for the bdellovibrio inhibition of algae (Burnham et al., 1976) the Myxococcus PC02 could be diluted 1/10, 1/100 and 1/1,000, and still achieve excellent lysis of a reasonably dense algal solution. Initially in the experiment these were 2.1×10^7 P. luridum cells per ml At day 4 the number of algae had fallen to 1×10^5 and at day 18 the number of algae had fallen between 5×10^5 and 1×10^6 for all inoculum concentrations. The number of Myxococcus

at 18 days was 1 x 10^6 for all inoculum concentrations. In this experiment a reasonable equilibrium appeared to be reached at about 1 bacterium per algal cell. This resulted in a significant decrease in turbidity measured at 630 nm (absorption maximum for P. luridum -phycocyanin). a result which would correlate with an improvement in water quality. The 0.1% inoculum took approximately 8 days to affect control over the algae which had grown significantl since the start of the experiment. However, once lysis started, the two microbial levels equalled those of the other flasks.

When Myxococcus PC02 was grown up in 0.2% tryptone and the cells removed by centrifugation, the resulting cell-free supernatant could be tested for anti-algal activity. When this was done only supernatants at 1:1 concentrations with P. luridum were able to show any lytic or inhibiting activity This result implies some extracellular enzymes are produced but for them to be active they must be in high concentration. My results suggest that this is accomplished via the clumping or ball formation around the host algae. This curve again illustrates the control activity that the Myxococcus PC02 cells have for these algae as there is a six-fold decrease in the absorbancy of the culture.

In testing the specificity of the Myxococcus PC02 lytic system a plate was prepared as illustrated in Fig. 7 (and as discussed above) for each of the algae listed below. The result, reported as lysis, is also listed:

1.	P. luridum var. olivacea	lysis	++++
2.	Plectonema boryanum	lysis	++++
3.	Oscillatoria sp.	some lysis	+
4.	Lyngbya sp.	some lysis	+
5.	Anabaena cylindrica	some lysis	++
6.	Symploca muscorum	no lysis	---
7.	Synechococcus sp.	lysis	+++
8.	Anabaena variabilis	lysis	+++

These results are preliminary but they do indicate a definite cross speciation is possible for lysis on agar surfaces. Species specificity has not yet been completely tested with the liquid culture system.

The micrographs shown in the appendix manuscript will provide visual description of the uniqueness of this system.

Legend for Figures

Figure 1. The relationship of P. luridum photosynthesis (ulO_2 produced per ml culture per hr) to the density of the culture (A_{630nm}) as measured in the mineral salts medium, AB.

Figure 2. The relationship of P. luridum photosynthesis to the density of the culture as measured in the proteinaceous medium 4xYP.

Figure 3. A comparison of photosynthetic ability between two lines of P. luridum. The "old" culture had been routinely transferred in my laboratory over 8 years. The "new" culture was obtained from the University of Texas Culture Collection only three weeks prior to testing. Both lines were grown in identical conditions. Culture densities were corrected to OD_{630} 1.0 in order to allow a precise comparison.

Figure 4. Effect of yeast extract on the growth ability of P. luridum cultures. A 20% inoculum into each medium from a 6-day old culture of P. luridum in AB was made at 0 days. Media as follows: ■ , AB control; ● , 1xYE (3g/l); ▲ 4xYE (12g/l).

Figure 5. Sephadex G25 column filtration of the bdellovibrio supernatant (BdS). The levels of P. luridum photosynthesis as affected by respective ml of filtrate are represented as are the corresponding levels of proteinaceous material present in the filtrate fractions. Photosynthesis was measured as in Burnham et al , 1976 using 1:1 ratios of P. luridum culture to the filtrate.

Figure 6. Effect of various substrates on the growth ability of P. luridum in AB medium. Control, AB only; BSA, Bovine Serum Albumin 100 ug/ml; Arginine, 200 ug/ml; polylysine, 100 ug/ml; lysine, 100 ug/ml; protamine, 100 ug/ml

Figure 7. Scanning electron micrograph of a 1 hr BdS-treated P. luridum illustrating the bleb structures on the trichome surface. The blebs in all P luridum cells treated with BdS appear to be randomly located and not positioned in any specialized region of the cells. Bar equals 1 um.

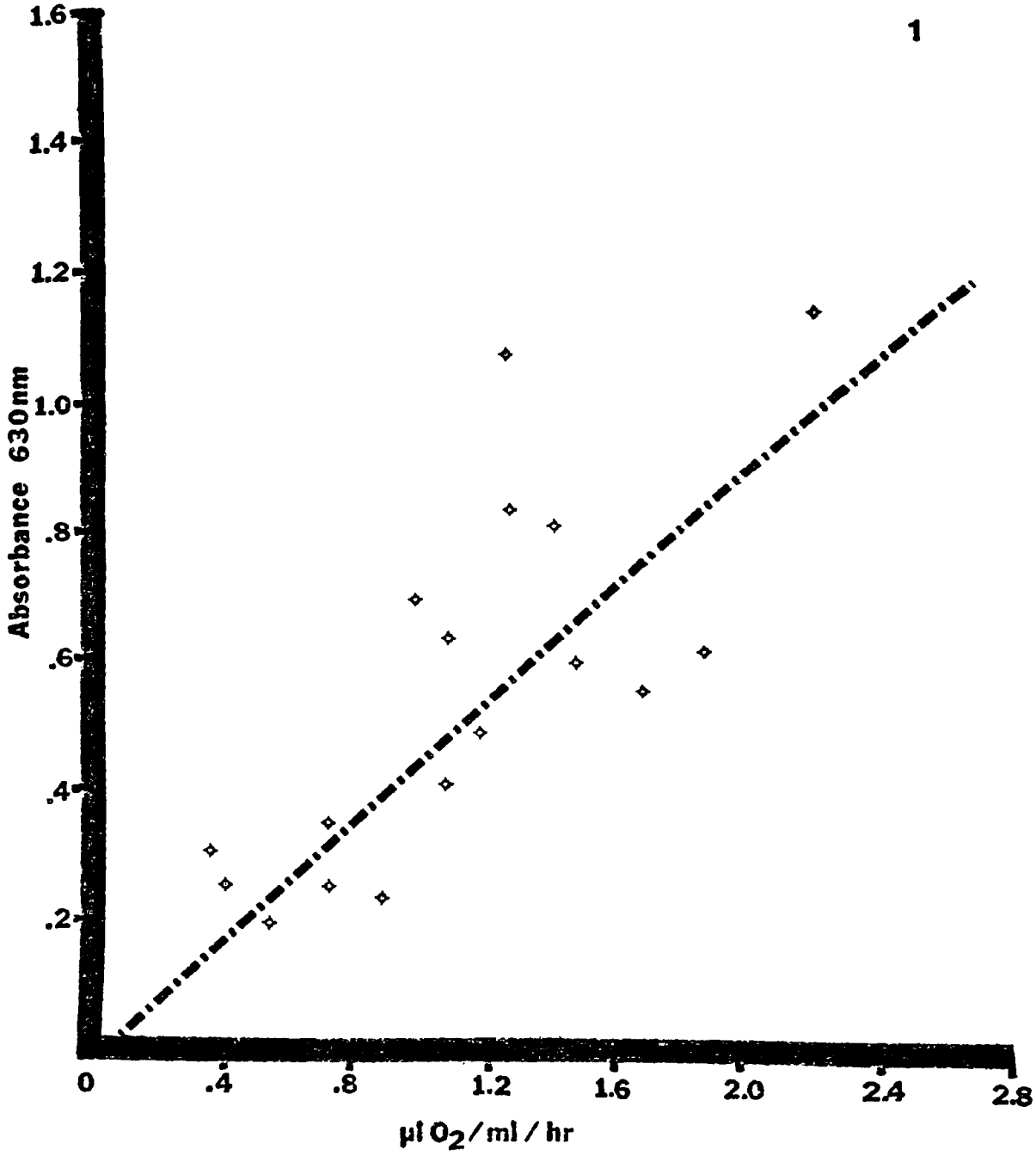
Figure 8. Graphs showing the results of long-term transfer experiments using P. luridum growing in shaking flasks at 30 C under continuously lighted conditions. The vertical line in all graphs illustrates the change when a 20% inoculum was transferred into a fresh flask containing only AB.

(a) The control curve for P. luridum alone indicates that no die-off or dilution of the algae occurred over the 32 days of the successive transfers. Similar survival and growth of the P. luridum was observed mixed with S. typhimurium and E. coli The curves were not shown as they are identical to that shown for P. luridum alone. When Myxococcus PC02 was added at 0 days the algae was not able to survive the transfer scheme and died out at 21 days.

(b) This curve represents the number of Myxococcus PC02 bacterial cells that correspond with the flasks shown in Fig. 10a. Following an initial increase in numbers and then fluctuated greatly. resembling the quantification of bacteria in AB alone.

(c) This curve shows that the presence of P luridum greatly increased the survival of S. typhimurium cells over the 32 days. Salmonella in AB alone were diluted out or died on day 17

(d) This curve illustrates that the presence of P. luridum only had a very slight effect on the survival of E. coli cells. Surprisingly the E. coli in AB alone managed to stay at 10^3 cells/ml in spite of no nutrient after 32 days.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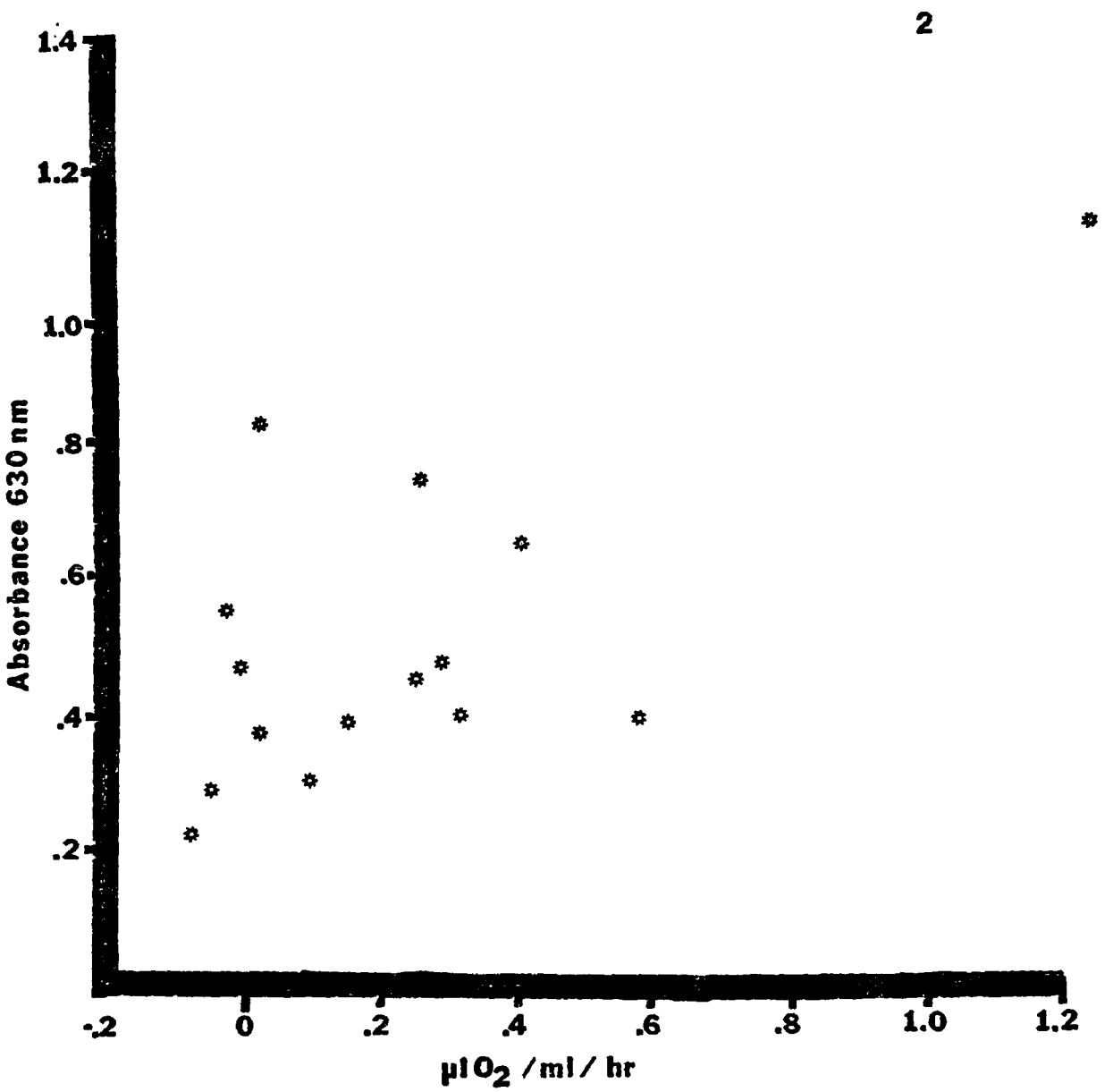
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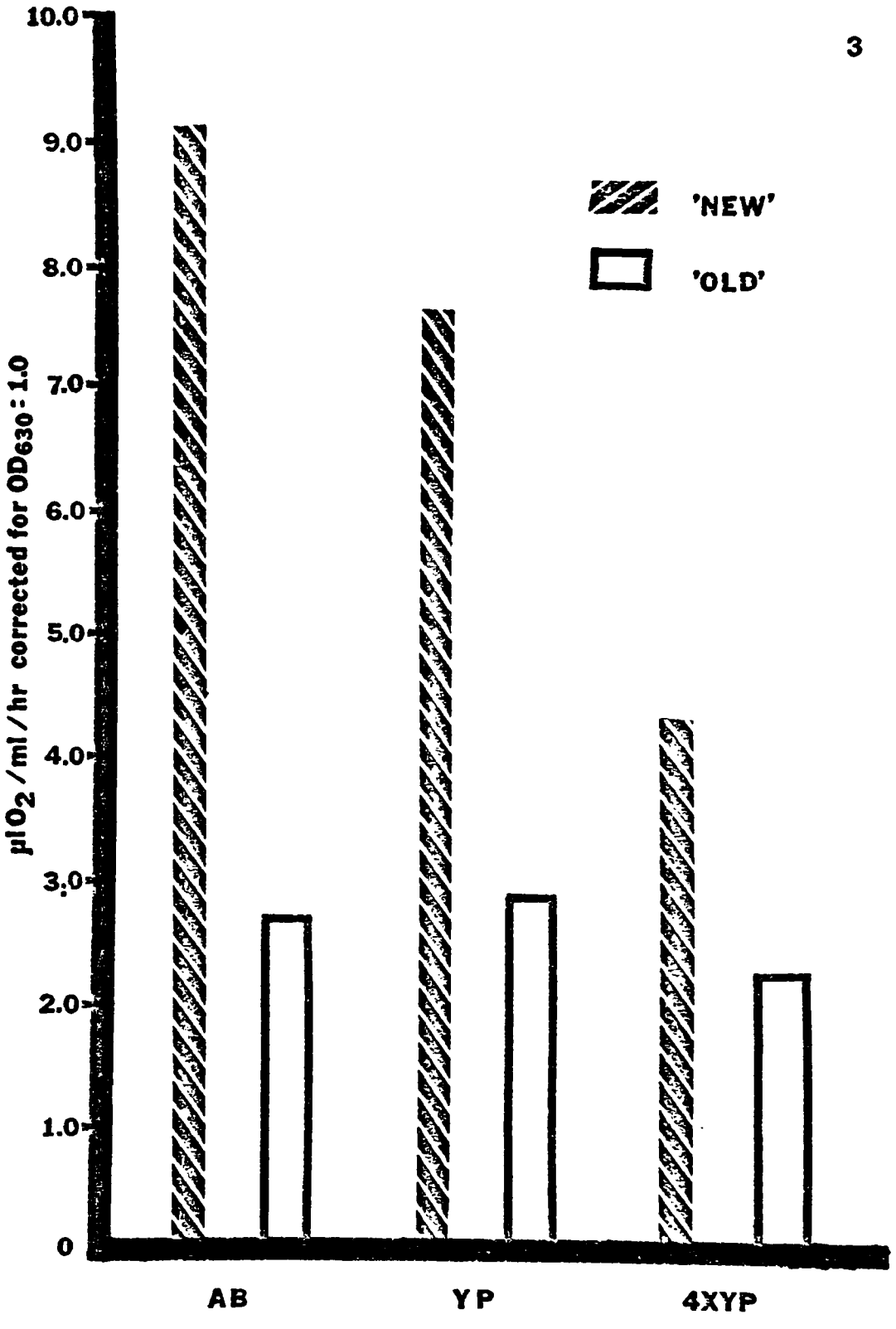
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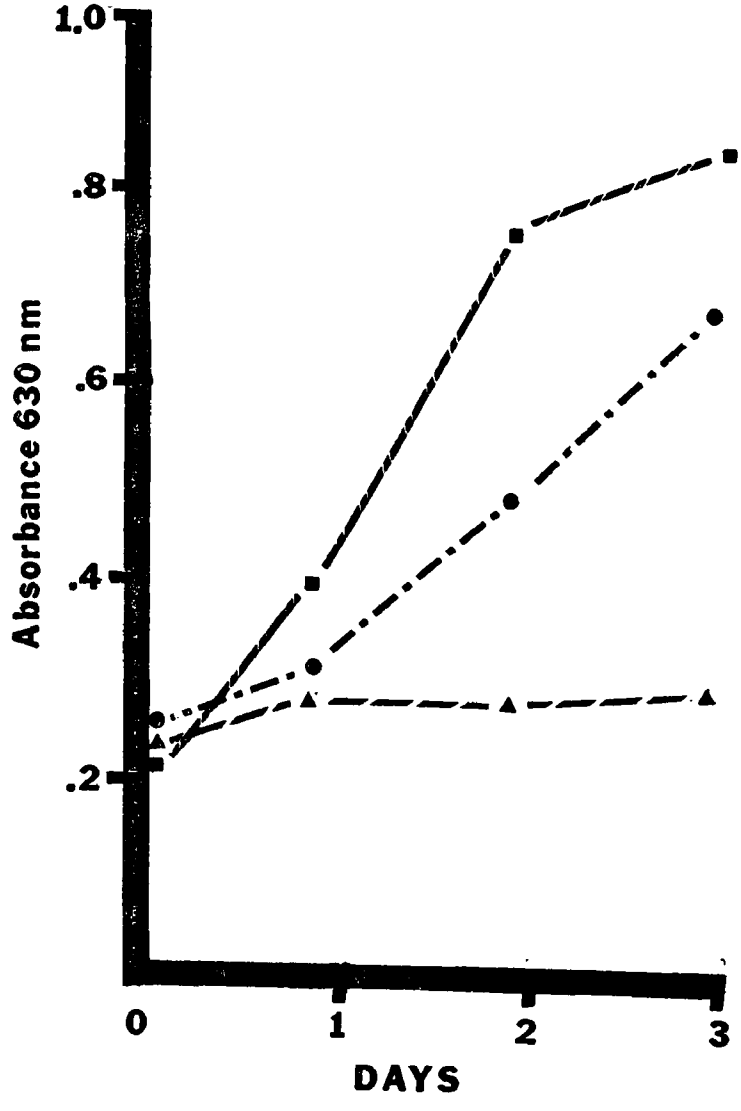
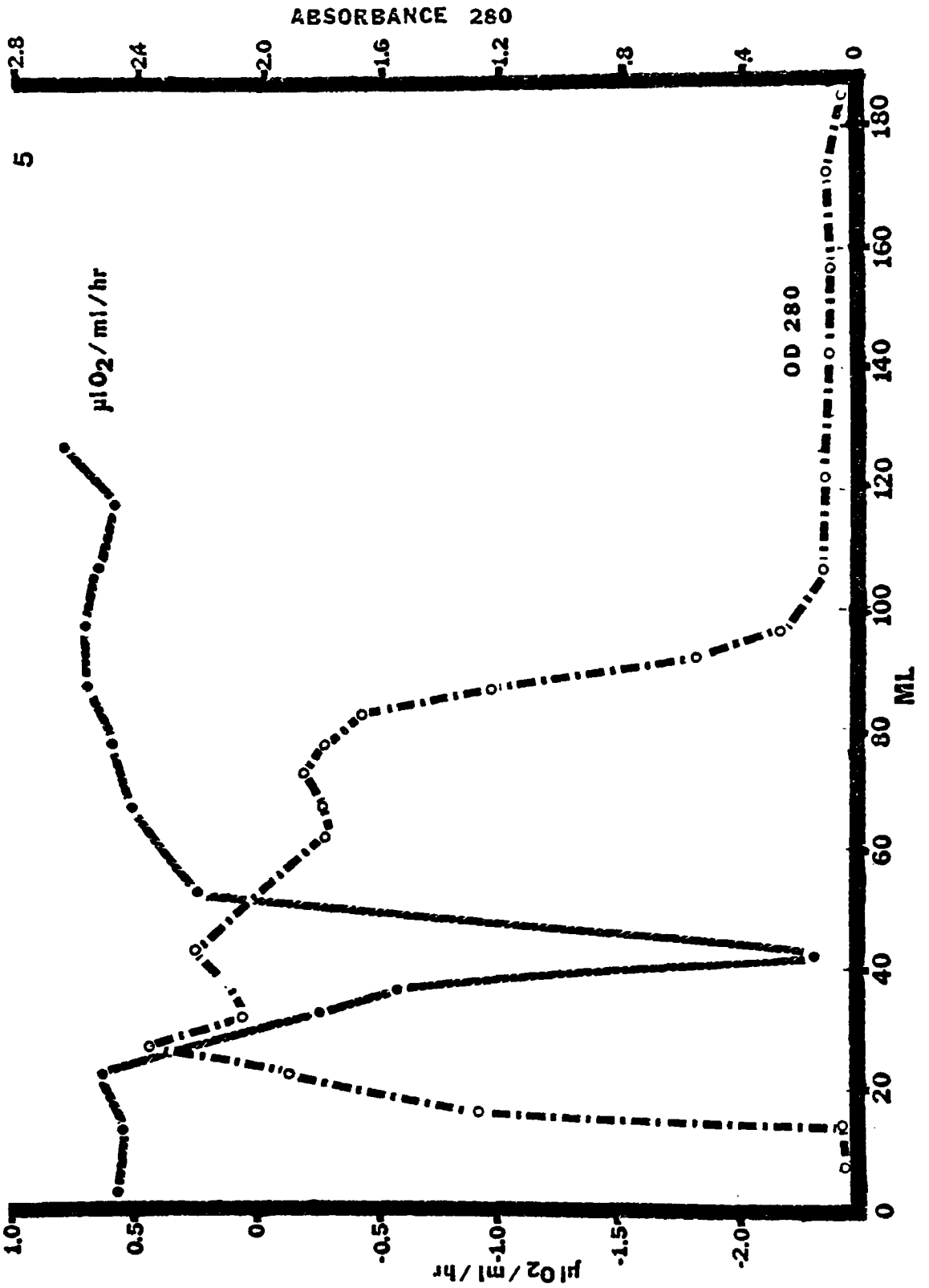
4

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1.2
.8
.4
0
5
μlO2/ml/hr
OD 280
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160
140
120
100
80
60
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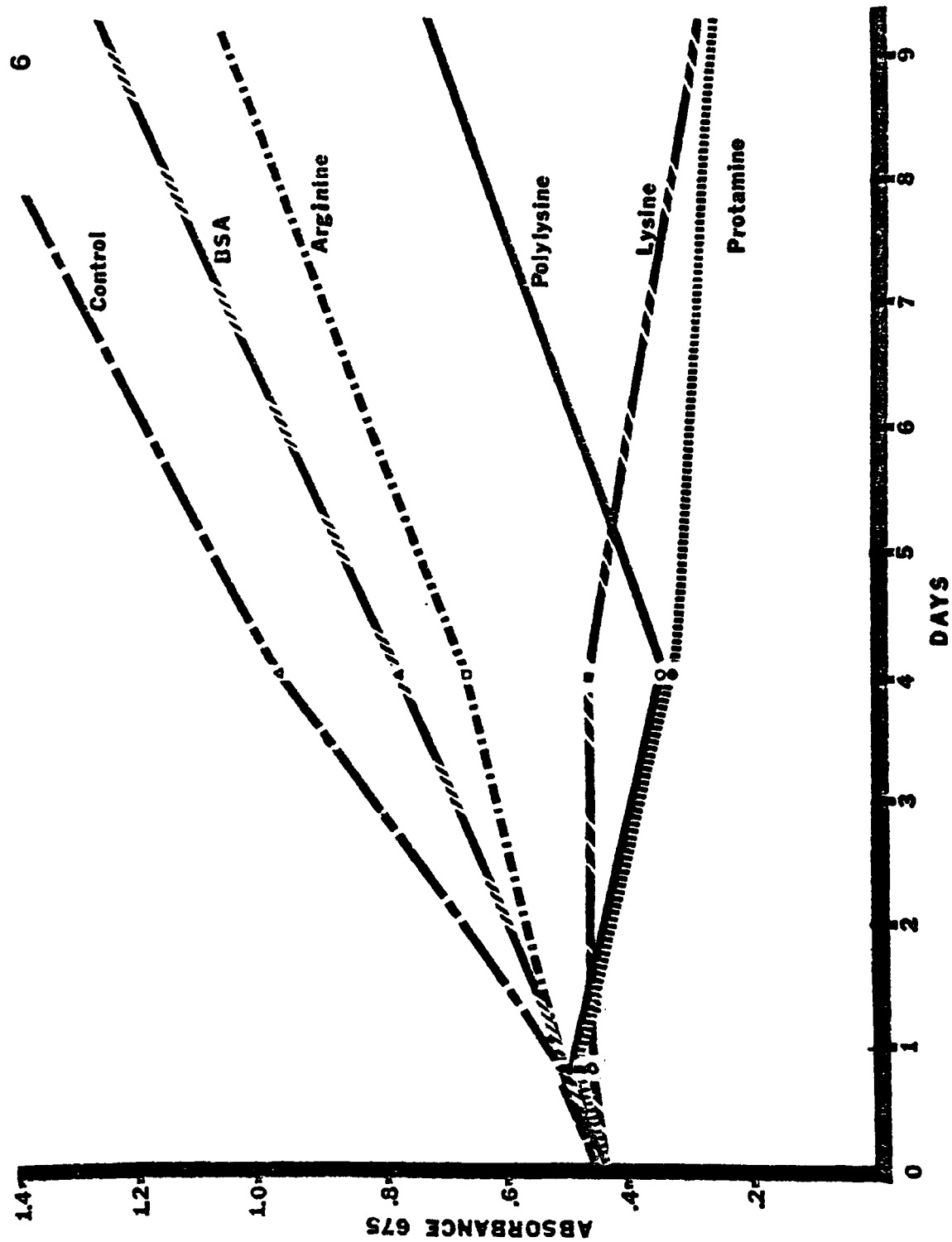
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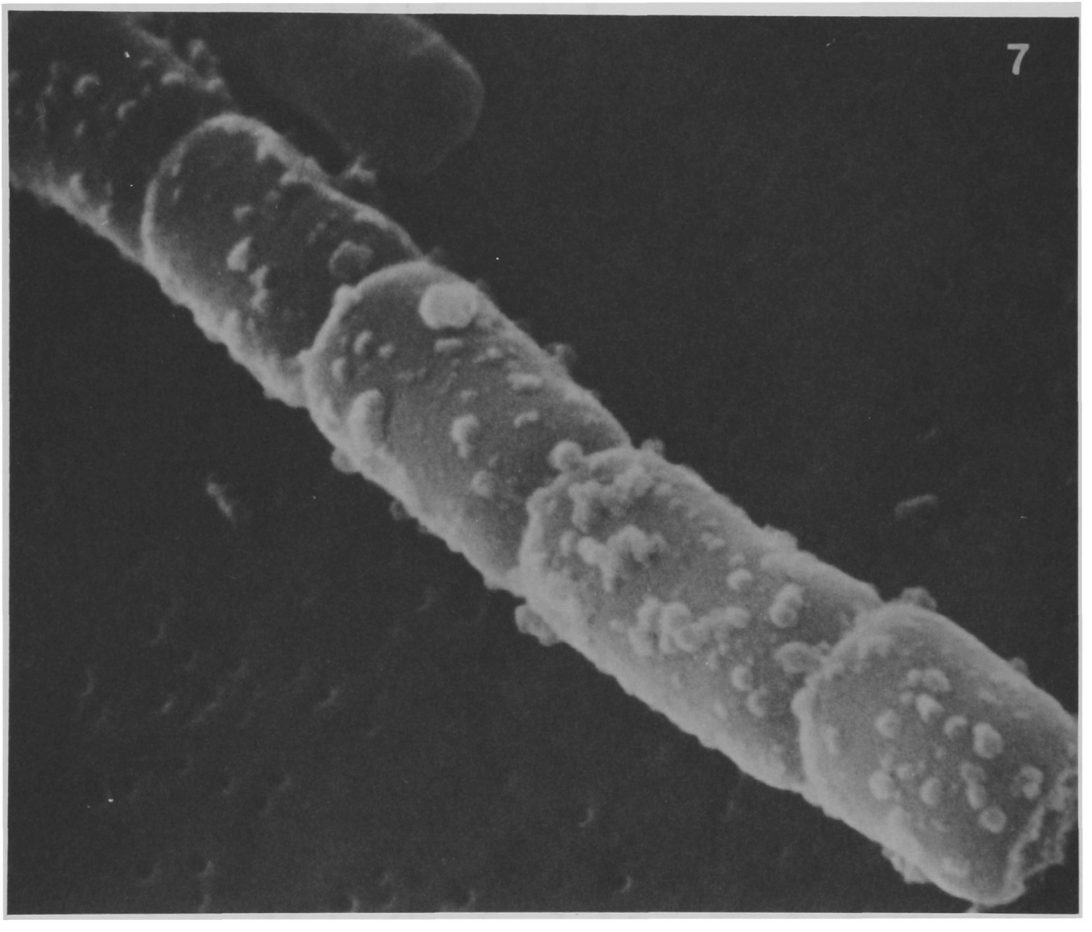
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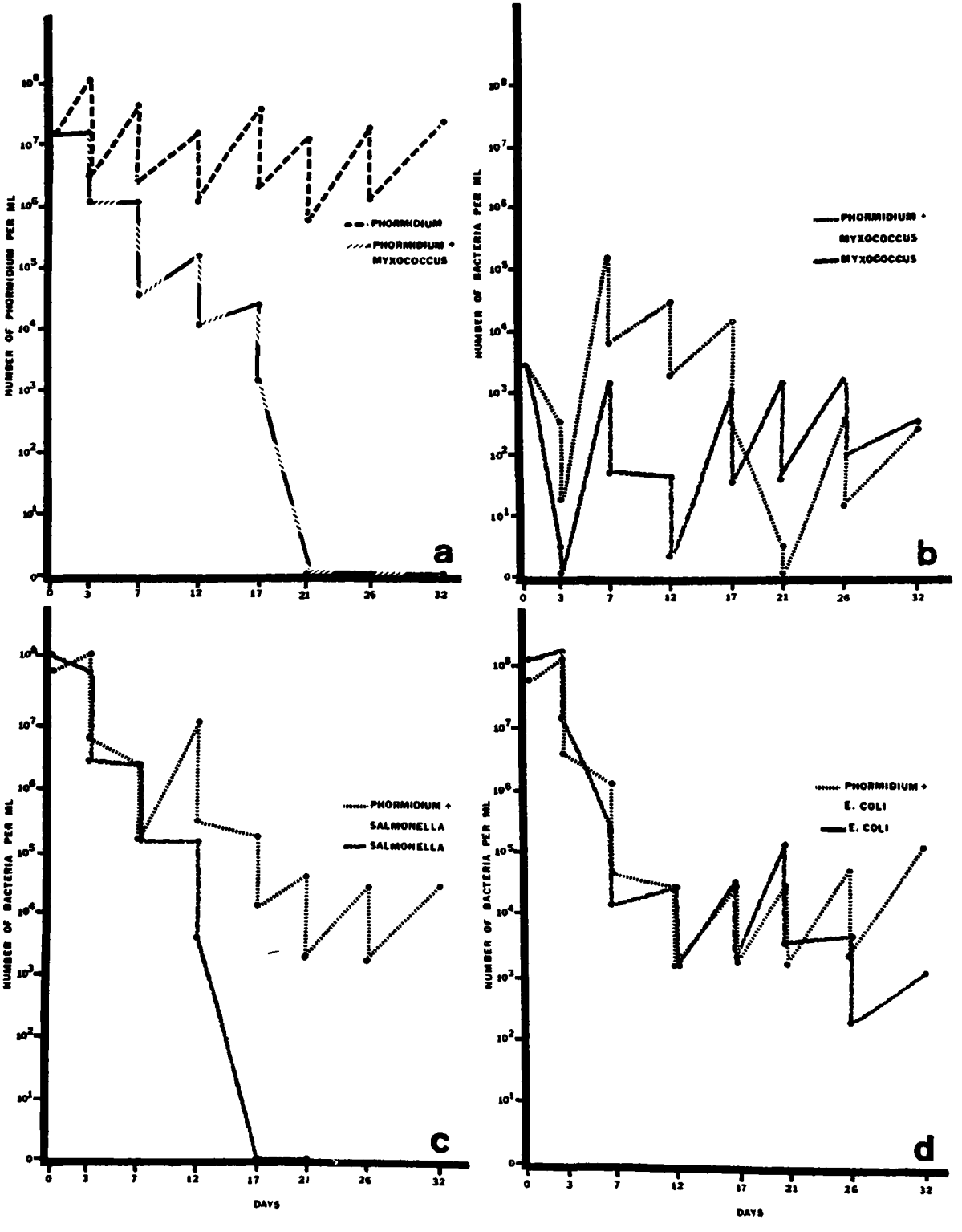
NUMBER OF PHORMIDIUM PER ML
PHORMIDIUM
PHORMIDIUM + MYXOCOCCUS
a
NUMBER OF BACTERIA PER ML
PHORMIDIUM + MYXOCOCCUS
MYXOCOCCUS
b
NUMBER OF BACTERIA PER ML
PHORMIDIUM + SALMONELLA
SALMONELLA
c
DAYS
NUMBER OF BACTERIA PER ML
PHORMIDIUM + E. COLI
E. COLI
d
DAYS

8

TABLE 1

Effect of suspension medium on the photosynthetic ability (n/O_2/ml culture/hr) of P. luridum

	1974	1975	1976	1977	1978	1979	n=
AB	+6.36	+4.97	+2.55	+1.69	+ .79	+1 30	2.94
YP	+5.94	+5.08	-----	+1.08	+ .74	+ .59	2.69
4xYP	+5.82	----	+2.11	+0.11	+0.02	-----	2.02
YP% of AB	93%	102%	-----	64%	94%	45%	91.5
4xYP% of AB	92%	----	83%	7%	3%	---	68.5

Table 2

Effect of Molecular Weight Fractionation on the Photosynthetic Inhibitory Activity of B. bacteriovorus Culture Supernatant Fluid

Fraction	Ps	SD	%Ps	%I
Control	2.99	± .49	100	0
Untreated	-1.57	± .34	- 52.5	152.5
50,000	-1.16	± .48	- 38.7	138.7
30,000	-1.25	± .33	- 41.8	141.8
10,000	-1.35	± .41	- 45.2	145.2
5,000	-0.19	± .09	- 6.4	106.4
1,000	+0.18	± .93	+ 6.0	94.0
500	+0.32	± 37	+ 10.7	89.3

Ps=ulO_2/ml P luridum culture/h; SD=standard deviation; $Ps= percent of control oxygen production; %I=percent inhibition of photosynthesis.

Table 3

Effect of Selected Compounds on P luridum Photosynthesis

Test	Percent Photosynthesis
P. luridum + AB	100
P luridum + peptone 12 g/l	52.5
P. luridum + BSA 12 g/l	21 7
P. luridum + casamino acids 14.4 g/l	77 7
P. luridum + arginine 12 g/l	100
P. luridum + lysine 12 g/l	80.9
P. luridum + lysine 0.2 g/l	98.6
P. luridum + yeast nucleic acids 12 g/l	46.7
P. luridum + yeast nucleic acids 5 g/l	97.5
P. luridum + yeast nucleic acids 1 g/l	108.3
P luridum + yeast nucleic acids 0.1 g/l	73.3
P. luridum + cAMP .01 M	69.0
P. luridum + cAMP .001 M	55.2
P. luridum + cAMP .0001 M	89.7
P. luridum + sodium citrate .5 g/l	23.9
P luridum + ammonium sulfate	128.3
P. luridum + K_2HPO_4 7 g/l	48.7
P. luridum + K_2HPO_4 2 g/l	34.5
P. luridum + $MgSO_4$ 0.1 g/l	58.4
P luridum + dextrose 12 g/l	87.5

PRESENT STATUS

The goal addressed inthis project, i.e., the development of an effective means of utilizing the bacteria to eliminate or control cyanobacterial populations in natural waters, is continuing to be addressed in my laboratory. Although the results described in this completion report for the interaction of Bdellovibrio bacteriovorus with cyanobacteria are discouraging toward any continuance of that effect, they have led directly to a most optimistic approach. I have now described both in the Results Section of the Completion Report and in two manuscripts contained within the Appendix of this report a novel predatory system for the control of unwanted cyanobacteria in water. This system utilizes the cooperative colonial growth of Myxococcus xanthus to ensnare and lyse and digest aqueous cyanobacteria. The system contains several advantages (Burnham, 1981) which should be reiterated here:

1. effective in autotrophic environment
2. utilization of dominant microorganisms as nutrient eutrophic aquatic systems
3. low inoculum of predator effective
4. independent of environmental agitation
5. non-specific host requirement
6. effective host entrapment mechanism
7. lytic system contained and segregated
8. multicomponent nature of lytic system
9. encystment ability of predator
10. predator survival in hostile environments

Major research is continuing on this Myxococcus predatory system partly funded by OWRT Grant #B-06-OHIO effective through September 30, 1982. My present plans are to join efforts with the principal other laboratory purusing this goal, i.e., that of Drs. William Stewart and Melvin Daft from the

Department of Biological Sciences, Queens College, University of Dundee, Dundee, Scotland. I will be working in their laboratory during a portion of 1981-82 on improving the predatory ability of the myxococci and concurrently expanding the host range of the system.

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RECOMMENDATIONS RESULTING FROM THIS PROJECT

1. Further research on the effect of Bdellovibrio bacteriovorus ATCC strain 15143 on cyanobacteria as a biological control mechanism should be stopped.

2. Research utilizing myxobacterial species as biological control agents should be increased. Data to date indicates potential success.

3. Continual search for undiscovered anticyanobacterial and antialgal bacteria should persist. For example Dr. Peter Hirsch, Professor, Institute for Microbiology. Kiel. Germany recently told me (unpublished results) that he had observed a Bdellovibrio-like bacterium that parasitized cyanobacteria but was unable to isolate it. Also the isolate described by Coder and Starr (1978) and Gronow and Mamkaeva (1980) "Bdellovibrio chlorellavorus" suggests that more ideal parasites which have adapted to aquatic habitats may be found in the future.

4. Research involving bacteria as microbial control agents needs to be simultaneously (1) developed in order to understand the nature of the biochemical and structural interactions that occur between predatory and prey or parasite and host; and (2) evaluated in increasingly complex systems which progressively mimic the natural ecosystems that are targeted (Burnham, 1981).

PERTINENT REFERENCES ORIGINATING FROM THIS GRANT PROGRAM

1. Burnham, J. C., S. A. Collart and B. W. Highison. 1981 Entrapment and lysis of the cyanobacterium Phormidium luridum by aqueous colonies of Myxococcus xanthus PC02. Archives for Microbiology. Accepted 1/81.

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APPENDIX

Entrapment and lysis of the cyanobacterium *Phormidium luridum* by aqueous colonies of *Myxococcus xanthus* PCO2

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Abbreviations: TEM, transmission electron microscopy; SEM, scanning electron microscopy; AB, algae broth; ABT algae broth plus 0.2% tryptone

ABSTRACT. A Myxococcus xanthus isolate from a farm drainage ditch, designated strain PCO2, is capable of rapidly inducing lysis of both agar and liquid-grown cultures of the cyanobacterium, Phormidium luridum, var. olivacea. Microscopic studies of the predator-prey interaction demonstrate that lysis of the cyanobacterium occurs within clumps and spherules formed by the cells of M. xanthus PCO2. In the earliest stage, one sees the formation of irregular microclumps of bacteria and cyanobacterial filaments. As these clumps mature, colonies 1 to 6 mm in diameter develops. The center of these densely green colonies contains cyanobacteria in various stages of degradation, while the periphery is almost exclusively a tightly woven mass of myxobacterial cells. Electron microscopy shows that long extrusions from the outer membrane of the M. xanthus PCO2 cells are involved in the formation both of initial clumps and of mature colonial spherules. These extrusions appear to efficiently entangle the cyanobacterial filaments in the culture environment. Predator-to-prey ratios of 1/10, 1/100 and 1/1000 have resulted in cyanobacterial lysis. Because the entrapment and lysis of P. luridum filaments by M. xanthus PCO2 appears to be independent of any other heterotrophic nutritional requirement, as well as of environmental agitation, this system has potential as a biological control technique for undesirable aquatic cyanobacteria.

Key words: biological control; cyanobacteria; electron microscopy; entrapment; lysis; Myxococcus; Phormidium; spherule

Although bacteriolysis has been studied for many years (Beebe, 1941), lysis of various species of cyanobacteria by bacteria was first reported in 1967 by Shilo. Since then numerous strains of gliding bacteria, principally members of the myxobacterial and cytophaga groups have been shown to be capable of lysing many cyanobacterial species (Wu et al , 1968; Shilo, 1970; Stewart and Brown, 1969, 1971; Daft et al., 1971, 1973, 1975; Christensen, 1977; Christensen and Cook 1978). Examination of the procaryotic lytic ability of these bacteria have resulted in the characterization of a variety of exoenzymes that are capable of hydrolyzing the bacterial cell wall, specifically, the peptidoglycan component (Ensign, 1965; Sudo and Dworkin, 1972; Haskå, 1974; Gnosspelius, 1978). Although equipped with the enzymatic means of lysing cyanobacteria, the various myxobacteria and cytophagas that have been described have not been effective in destroying cyanobacterial populations in dilute agitated liquid environments. In addition, aqueous cyanobacterial lysis by members of Myxococcus groups is unusual as these groups generally do not grow well in a liquid medium, preferring a solid substrate upon which to develop (Schürmann, 1967).

This paper describes a unique myxobacterial lytic system in which colonial encapsulation of entrapped cyanobacteria leads to long-term survival of predatory myxococci in aqueous environments where the only heterotrophic nutrient sources are the cells and byproducts of autotrophic cyanobacterial growth.

As previous reports on the cyanobacterial lytic bacteria have described (Stewart and Brown, 1969; 1971; Daft et al., 1971; 1975), our isolates (Burnham et al., 1980) are being screened carefully to determine their potential as cyanobacterial control agents. The data presented in this paper indicates that the M. xanthus PCO2 isolate has several important characteristics which heighten this potential.

MATERIALS AND METHODS

Isolation and Identification

The M. xanthus PCO2 was isolated from a grab sample obtained from a roadside ditch on Route 2, Port Clinton, Ohio. The sample (0.5 ml) was spread on a 96 hr lawn of the cyanobacterium Phormidium luridum. After several days, the largest region of cyanobacterial lysis was picked with a sterile loop, placed on a new lawn of P. luridum. Typical spreading cultures of myxobacteria were picked and isolated on an algae agar medium (Difco) containing 0.2% Bacto tryptone (Difco).

The bacterial strain thus isolated was identified as a Myxococcus xanthus species based on the criteria of Zahler and McCurdy (1976), using the characteristics of yellow to yellowish-orange vegetative cell masses, a myxospore diameter of 1.8 μm, and the initial ability of this strain to form small fruiting bodies without a stalk. As will be shown in this paper the strain continues to produce a fruiting structure on the surface of aqueous grown colonies. The strain was designated M. xanthus PCO2 based on its site of origin.

Organisms - The cyanobacterium used in this study, Phormidium luridum var. olivacea (No. 426) was obtained from the University of Texas Culture Collection of Algae and is maintained bacteria-free. The comparator strain of Myxococcus xanthus, strain 25232, was obtained from the American Type Culture Collection. The P. luridum was routinely maintained using Difco algae broth (designated AB) as previously described (Burnham et al., 1976). The myxococci were routinely maintained axenically in Difco algae broth containing 0.2% Bacto tryptone (designated ABT) as well as being continuously grown on mature lawns of P. luridum that had been grown on Difco algae agar The axenic myxococci were grown at 30C on a rotary shaker at 100RPM. Myxospore formation was induced by suspending 3-day old ABT grown PCO2 cells in 2% casitone and 0.05% $MgSO_4$ broth containing 0.2M glycerol (Dworkin and

Gibson, 1964). shaken rapidly at 24C and observed periodically by phase contrast microscopy.

Mixed cultures M. xanthus PC02 was centrifuged from ABT cultures, washed twice in algae broth and then suspended in AB and inoculated into 4 to 7-day-old P. luridum cultures grown autotrophically in AB. Predator-to-prey ratios of 1/10, 1/100 and 1/1000 were utilized. Enumeration of the P. luridum was accomplished as previously described (Burnham et al., 1973) with a Petroff Hausser Chamber with a volume constant of $2x10^7$ M. xanthus enumeration was accomplished by homogenizing the aqueous myxococcal sample into AB with a glass tissue grinder followed by dilution and plating on ABT containing 1.5% agar Myxococcal colonies were counted after 7 days of growth. The mixed culture interactions were conducted at 30C in 500 ml side arm Erlenmeyer flasks on a rotary shaker under 3200 lx in a cycle of 16 h light-8 h dark. For long-term experiments, actively lysing mixed cultures were maintained by successive 1% transfers into fresh cyanobacterial cultures.

Light Microscopy - Myxococcus colonial spherules, formed either axenically in ABT or in AB with P. luridum, were gently removed from the growth flask by pipette and examined by bright field phase contrast microscopy with a Zeiss Axiomat Microscope using quartz halogen illumination. For parafin embedding, the spherules were fixed in 10% phosphate buffered formalin (Fisher) for 2 h, dehydrated in ethanol and embedded in parafin. The parafin sections were Gram stained.

Electron Microscopy - In preparations for TEM, M. xanthus PC02 cells and spherules were fixed with a standard bacterial fixation (Burnham et al., 1968) using 4% glutaraldehyde followed by 2% OsO_4. Following fixation, specimens were embedded in Epon 812, stained with both uranyl acetate and Reynolds lead citrate, and photographed in a Philips 300 Transmission Electron Microscopy operating at 60 KV.

For SEM, all cells and spherules were directly fixed in 4% glutaraldehyde in 0.1M KH_2PO_4 at pH 7.2 for 12 h. Cells and small spherules were filtered onto 0.22 um or 0.45 um Nucleopore membrane filters and then dehydrated in ethanol as recommended by Hayat (1978) and critical point dried with CO_2 in a Polaron (Watford, England) apparatus.

Larger colonial spherules were dehydrated and critical point dried in baskets and then applied to aluminum stubs using double-sided Scotch brand tape. All specimens were coated with gold-palladium in a Polaron SEM Coating Unit E5100 and examined in a Cambridge 180 scanning electron microscope.

RESULTS

Initial myxobacterial isolates were identified by plaque formation on cyanobacterial lawns following seeding with water from various natural sources. The M. xanthus PC02 isolate formed spreading plaques of cyanobacterial lysis which increased by approximately 0.5 cm in diameter per day (Fig. 1). When examined by phase contrast microscopy, the vegetative myxococcal cells were commonly between 4 to 7 μm in length and 0.5 to 0.8 μm in diameter (Fig. 2). Although early agar grown cultures of the M. xanthus PC02 routinely yielded fruiting structures, this character was lost with serial transfer. Of special interest was the discovery that this strain aggregated to form myxospore bearing spikes on colony surfaces when grown in liquid cultures (see Fig. 10). As has been shown for other Myxococcus species (Dworkin and Gibson, 1964), the spore forming characteristic of this strain also could be induced by treating vegetative rods with solutions of glycerol

Microscopic examination of plaques on P. luridum lawns revealed M. xanthus PC02 cells in various stages of myxospore formation. Predominantly short rods were present with only a few refractile oval myxospores seen. Examination of the lytic edge of the plaque disclosed intermingled M. xanthus PC02 rods and P. luridum cells with the cyanobacterial filaments in various stages of degradation.

The unique nature of the PC02 strain became apparent when flocculation and cyanobacterial lysis was found to occur rapidly in agitated liquid environments following inoculation of these myxobacteria into autotrophically grown P. luridum (Fig. 3). The initial floccules usually appeared very irregular in shape; however, with age (24 h or more) and continuous rotary or reciprocal agitation, the clumps that contained cyanobacteria often became quite spherical or ellipsoidal, often achieving a diameter of 1 to 6 mm (Fig. 3b)

Additional evidence of the unique nature of the M. xanthus PC02 strain was obtained by comparison with the ATCC strain M. xanthus 25232. When this strain was used in place of the M. xanthus PC02 strain, only small plaques formed on P luridum lawns. Furthermore, when grown axenically in ABT, this strain exhibited dispersed growth with few flocculations. Finally, in AB grown P luridum cultures, M. xanthus 25232 caused very little lysis and no significant clumping.

When a 1% inoculum of washed M. xanthus PC02 was added to an AB-grown culture of P. luridum there was a distinct alteration in the appearance of the culture over 48 hr It underwent a sequential change from a uniform dense green to a dense green flocculation to a translucent yellow. When examined microscopically, this translucent medium contained many aggregates of yellow-orange pigmented myxococcal cells.

Figure 4 demonstrates the turbidimetric reduction resulting from increasing inocula of M. xanthus Pc02 into dense 6-day cultures of P luridum (approximately 10^7 cells/ml). Growth of the P. luridum supplemented with 10% ABT medium exceeded 2.0A at 630 nm, the absorption peak for the photosynthetic pigment, phycocyanin, in about 4 days. Cultures which were inoculated with 1% and 10% inocula of myxococci (predator-to-prey ratios of 1:100 and 1:10, respectively) were nearly cleared in 48 hours. Some cycling occurred over long-term incubation, but renewed myxococcal lysis of the cyanobacteria would return the absorbance to a level between 0.1 and 0.2A (about $1x10^5$ cyanobacteria per ml). Lower myxococcal inocula at predator-to prey ratios of 1:1000 or lower took longer to affect the cyanobacterial population. Figure 3 shows that with a 0.1% inoculum lysis occurred rapidly after 8 days of lag time. Once lysis occurred, the cycling pattern of cyanobacterial growth and subsequent lysis resembled that occurring in cultures with higher initial levels of myxococci.

When the flocculating processes and myxococcal colony formation was examined by phase contrast microscopy. a distinct pattern was revealed. Figures 5a, b show the early stages of flocculation in which relatively few myxococci are able to bind together several cyanobacterial trichomes. The myxococi gradually ensnare increasing numbers of P. luridum and themselves begin to multiply forming a peripheral encapsulation of the cyanobacteria. Fig. 5c illustrates that this encapsulation can occur very rapidly when high myxococcal inocula are employed. When our standard 1% inoculum is introduced into a 6-day old cyanobacterial culture, a well-developed thick capsule of myxococci develops around a core of trapped P. luridum in 96 h.

Mature clumps often take the form of spherules or small ellipsoids with a distinct inner and outer morphology (Figs 5d, f). The time course of forming these spherules depends upon the number of myxococci present per ml in the aqueous environment in question.

At 1% inocula, or at about 10^5 myxococci per ml, it takes roughly 48 h to 96 h for mature spherules to form (Fig. 5d, f) but at 100% inocula, or about 10^7 myxococci per ml, lytic spherules are formed in less than 1 h (Fig. 5c).

Figure 5e shows that the earliest sign of degradation of the P. luridum as detected by light microscopy is the separation of a trichome into short filaments and single cells. Following this, within the core of the spherule, the cyanobacteria swell and lyse and all that remains is an amorphous area intermixed with spore and vegetative rod forms of the myxococci

Figure 5f shows a spherule with a myxococcal encapsulation of over 50 μm in thickness surrounding a core with a diameter of 150 um. These spherules have a unique beauty to them when examined by light microscopy. The inner structure of the spherule appears green in color because of the chlorophyll a and phycocyanin pigments from the P. luridum. On the other hand, the outside of the spherules are a dark yellow-orange in color imparted by the pigment of the myxococci

When these spherules are examined by scanning electron microscopy, the unique architecture of the surface of the spherule becomes evident. The colonial form of this strain very commonly assumes a spherical shape as shown in Fig. 6. This spherule, with its diameter of about 1 mm, is evenly convoluted over its entire surface. Colonies appear to exhibit marked variability in the number and size of these convolutions. However, there is a correlation with aging. As colonies age, the height of the convolutions on the spherule surface become more accentuated (Fig. 10). When the convolution is examined under a higher magnification (Fig. 7), the individual rod-shaped M. xanthus PCO2 cells that form the convolution become apparent. Closer observation of the cells comprising the colonial surface reveals many fibrous extrusions extending out from the cell walls of the bacteria (Fig. 8). The arrow demonstrates the entanglement that can occur as these cells form the outside layers of the spherule. These convoluted surfaces on spherules form either during predatory growth with cyanobacteria or in axenic culture on ABT.

Examination of thin sections of these surface fibers by TEM confirms their site of origin to be the outer membranes of the gram-negative myxococcal cell (Fig. 9). These fibers are actually tubes of an average diameter of 25 nm formed by lengthy extrusions of the outer membrane into the cell's immediate environment.

As will be discussed later, our present hypothesis of cyanobacterial lysis involves the concept that the myxococcal cells move upon the colonial surface. This idea is supported by sequential observations of colonial spherules either in axenic culture or with cyanobacteria. The surface convolutions shown in Figs. 6, 7 develop into spiked configurations of various shapes and sizes (Fig. 10). This appears very analogous to the swarming and fruiting body forming abilities previously described by Dworkin, 1973; Hodgkin and Kaiser, 1977; and Kaiser et al., 1979 which involve a coordinated

gliding movement of the myxococcal vegetative rods.

The examination of stained parafin sections of these colonial forms emphasizes that the dense myxococcal exterior of the spherules provides a distinct separation of the interior environment of the spherule from the aqueous habitat in which the organisms are grown. This is extremely important to our hypothesis describing the mechanism of the cyanobacterial lysis by these colonies and will be discussed later in this chapter.

Figure 11 manifests another form that the surface of these colonies can assume. These colonies, formed after the addition of myxococcal cells (10^5/ml) to P. luridum (10^7/ml). exhibit a surface with many ridges. A closer examination of the surface of one of these 5-day old spherules show filaments of the P luridum caught in a mass of myxococcal cells (Fig. 12). Also visible are myxococci in various stages of encystment (arrows). This micrograph shows many myxococcal vegetative rods to be in a longitudinal orientation relative to one another along the ridges on the spherule surface.

When thin sections of a young predatory myxococcal colony are observed by TEM, various zones can be described. The periphery of the colonial spherule is comprised primarily (Fig. 13) of myxococci with their numerous outer membrane extrusions. The zone just within this myxococcal periphery (Fig. 14) contains a large number of both cyanobacteria and myxococci intermixed. No specific orientationof the myxococci to the cyanobacteria has been observed. Fig. 14 shows that a significant amount of lysis can be observed in this region as many cyanobacterial membraneous skeletons are seen along with apparently normal cyanobacterial The central regions of the core are illustrated by Fig. 15. Lysed cyanobacteria predominate as the space is primarily occupied by thylakoidal skeletons. We have noticed in many sections of older predatory colonies that myxococcal cells can often be found within these skeletons as shown in Fig. 16. This condition is only observed with cyanobacterial skeletons suggesting that the myxococcal

cells enter only previously lysed cyanobacterial cells and are not endoparasitic in the manner of Bdellovibrio bacteriovorus (Burnham et al., 1976)

Two experimental approaches were utilized to provide additional information on the exoenzymes produced by the myxococcal colonies. The first measured the lytic ability of a cell-free myxococcal culture supernatant fluid. A 5-day old ABT supernatant fluid was added to a culture of P. luridum as described in studies of Bdellovibrio bacteriovorus' lysis of cyanobacteria (Burnham et al., 1976). No lysis or absorption decrease at 630 nm occurred with volume ratios of less than 1:1, supernatant fluid to cyanobacterial culture. At a 1:1 mixture a 50 percent drop in 630 nm absorption was measured after 5 days of interaction.

Second, we utilized a membrane separated, two-chambered culture apparatus to determine if P. luridum lysis could occur when the cyanobacteria were physically separated from the myxococcal colonies. The culture apparatus, manufactured by TechniLab contained two chambers separated by a Millipore Corp. membrane filter containing 0.2 μm pores (see Fig. 17). We tested the ability of large molecules to pass across this membrane by placing blue dextran dye (Sigma Chemical Co., MW 2,000,000) in one chamber and measuring its concentration equilibration with the opposite chamber. Under agitated conditions as described for myxococcal interaction in the Materials and Methods, dye equilibration occurred in less than 24 h, proving that any myxococcal exoenzyme should have no difficulty crossing the membrane barrier. Experiments were set up so that each chamber would contain P. luridum at 10^7 cells per ml. One side, shown in Fig. 17b, contained an equal number of 3X washed myxococci. These myxococci proceeded to flocculate and lyse the cyanobacteria on their side of the membrane barrier The P. luridum on the non-myxococcal side were unaffected by any myxococcal exoenzymes which crossed the 0.2 μm pore separating membrane (Fig. 17a) Fig. 18 shows the level of chlorophyll a to increase normally in the chamber containing only

P. luridum. In contrast, the side containing both N. xanthus PCO2 and P. luridum showed a complete loss of measurable chlorophyll a after 42 h.

DISCUSSION

Many papers have described gliding bacteria that are capable of lysing cyanobacteria: i.e., several myxobacterial strains (Daft et al , 1971, 1973, 1975; Shilo, 1970; Stewart and Brown, 1971 and Wu, 1968) and two Cytophaga strains, N-5 (Stewart and Brown, 1969) and a Cytophaga sp. (Shilo, 1967). The strain described by Shilo, 1970, designated myxobacter FR-1, the strains of Stewart and Brown, 1971, designated myxobacter 45 and 46, the myxobacter AL-1 strain of Ensign and Wolfe (1965) and the strain used by Daft and Stewart (1971), CP-1, appear to be members of a newly designated genus of non-fruiting, high G-C gliding bacteria, Lysobacter (Christensen, 1977; Christensen and Cook, 1978). None of these papers have reported lysis via a colonial aggregate mechanism as described in this paper.

The natural aqueous environment poses three major problems to all predatory myxobacteria: (1) encountering a susceptible host; (2) maintaining a high enough concenrationof the lytic enzyme to achieve the destruction of the cyanobacteria host; and (3) retaining a high enough concentration of the liberated nutrients to allow myxococcal growth. The myxococcal aqueous colony system we have described provides a distinct separation of the lytic and nutrient containing environment from the external environment of the colony and provides a large adherent surface upon which to gather susceptible prey.

Rosenberg et al., 1977 described the phenomenon of cooperative growth used by M. xanthus FB to hydrolyze a complex protein (casein) in aqueous culture. By increasing the myxococcalcell density in an environment it was shown that the growth rate also increased. The production of an extracellular protease was necessary for growth but the rate of growth was independent of the rate of protease concentration. The important finding was that with

increasing cell densities the intercellular distances were reduced sufficiently to allow a more effective utilizationof the dispersed proteases and hydrolysis products of casein needed for the myxococcal growth. The authors agree with Dworkin, 1972 that the myxobacterial life cycle may be specifically adapted to maintain the high cell densities needed for efficient growth in natural environments. The aquatic myxococcal colonies described in this paper appear to be an excellent example of the use of the cooperative growth phenomenon via the close intercellular associations maintained by the myxococci in the peripheral retion of the colony.

The aggregative nature of the myxococci has been well described (Dworkin 1972, 1973; Kaiser et al., 1979) so it should not be surprising to find such colonial forms in liquid environments. Burchard (1975) described a mutant of M. xanthus which in axenic liquid culture formed colonial spherules that closely resemble those described in this report. On a solid substrate, this agtregation is effected by the gliding motility of the Myxococcus. In liquid environments, this may depend more upon the interaction of bacterial surface fibrils such as the myxococcal fimbriae described by Dobson et al , 1979, or the outer membrane extrusions that are clearly evident in our micrographs. An adherent role for these surface appendages is suggested by the ability of the myxococci to rapidly flocculate a dense population of cyanobacteria if added in an equal or greater number with respect to the prey.

The formation of colonial spherules and related shapes appears related not only to this initial flocculation ability but also to the ability to continually ensnare cyanobacteria and transport them to the core of the colony. We believe that the gliding motility of the myxococci is involved. Although no direct in vivo observations of swarming on the surface of the colonies has yet been attempted, observations of the progressive formation of surface "spikes" (Fig. 10) suggests a very motile, constantly shifting

surface population. This surface swarming of the myxococci could gradually shift the cyanobacteria to the center of the core by continually covering over the captured cyanobacteria. Once the cyanobacteria reach the core they appear to be lysed by enzymes that act on their cell walls (Fig. 15) since the peptidoglycan layer is consistently absent from the P. luridum skeletons.

It has been suggested that both surface-bound enzymes and extracellular lytic substances are responsible for the bacterial lysis of cyanobacteria (Shilo, 1970; Daft and Stewart, 1971). Early interactions (Figs 5a, b) suggest direct adherence but observations of older colonies suggest exgracellular lysis (Figs. 5e, 14, 15). The Myxococcus genus has been shown to produce a variety of bacteriolytic enzymes such as proteases (Gnosspelius, 1978), a glucosaminidase (Sudo and Dworkin, 1972). a D-alanyl-N lysine endopeptidase (Sudo and Dworkin, 1972) as well as antibiotics (Rosenberg et al., 1973). Each is capable of acting on the cell walls of cyanobacteria which contain peptidoglycan as their major structural component (Lang, 1968) This arsenal of anticyanobacterial substances can be trapped and thus concentrated within the core region by the thick encapsulating layers of myxococcal cells making up the outside of the colony. The resultant lysis of the entrapped cyanobacteria releases nutrients which then may be taken up by the myxococci for their own growth leading to further enlargement of the colony

The myxococcal strain described in this paper is one of several which we have isolated, and which are capable of cyanobacterial predation (Burnham, 1980). Although each has distinguishing characteristics (unpublished data). all of them are capable of growing with no heterotrophic nutrient present other than the cyanobacteria or extracellular substances produced before the lysis of the cyanobacteria.

The formation of colonial aggregates as described in this paper would offer a distinct survival advantage to these myxococci It would allow the

utilization by these M. xanthus strains of an abundant heterophic nutrient source, i.e., the cyanobacteria that proliferate in eutrophic waters, which at the same time, are a very inaccessible nutrient source to the majority of aquatic microorganisms. The nutritional advantage to the myxococci as well as their demonstrated effectiveness in lysing the cyanobacterial cells of P. luridum growing in agitated autotrophic aqueous environments, clearly is of broad biological interest. Furthermore, the discovery of these strains suggests a new and exciting approach to the biological control of unwanted cyanobacterial populations.

ACKNOWLEDGEMENTS

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Legends for Figures

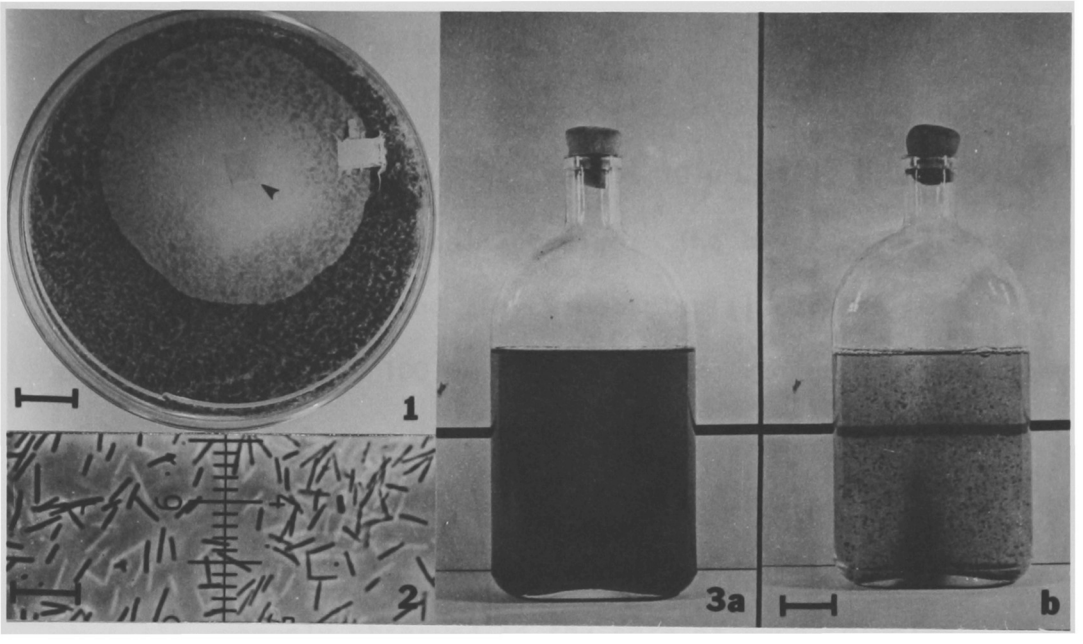
PLATE ONE

Fig. 1. An AB agar plate showing a spreading plaque of *M. xanthus* PCO2 on a growing lawn of *P. luridum*. The inoculation site is shown by an arrow. Bar equals 1.0 cm.

Fig. 2. Phase contrast micrograph of rod-shaped vegetative cells of the *M. xanthus* PCO2 strain. Bar equals 10 μm.

Fig. 3. Flocculation of a cyanobacterial culture by *M. xanthus* PCO2. a) Control: 8-day culture of *P. luridum* grown in autotrophic AB medium. b) Identically grown 8-day culture to which 1 ml of a 48 h ABT culture of myxococci was added on day 6. Bar equals 2 cm.

Fig. 4. Clearing effect of *M. xanthus* PCO2 on a population of 10^7 *P. luridum* cells/ml A 48 h culture of *M. xanthus* PCO2 in ABT (10^7cells/ml) was used for the three inocula.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3a
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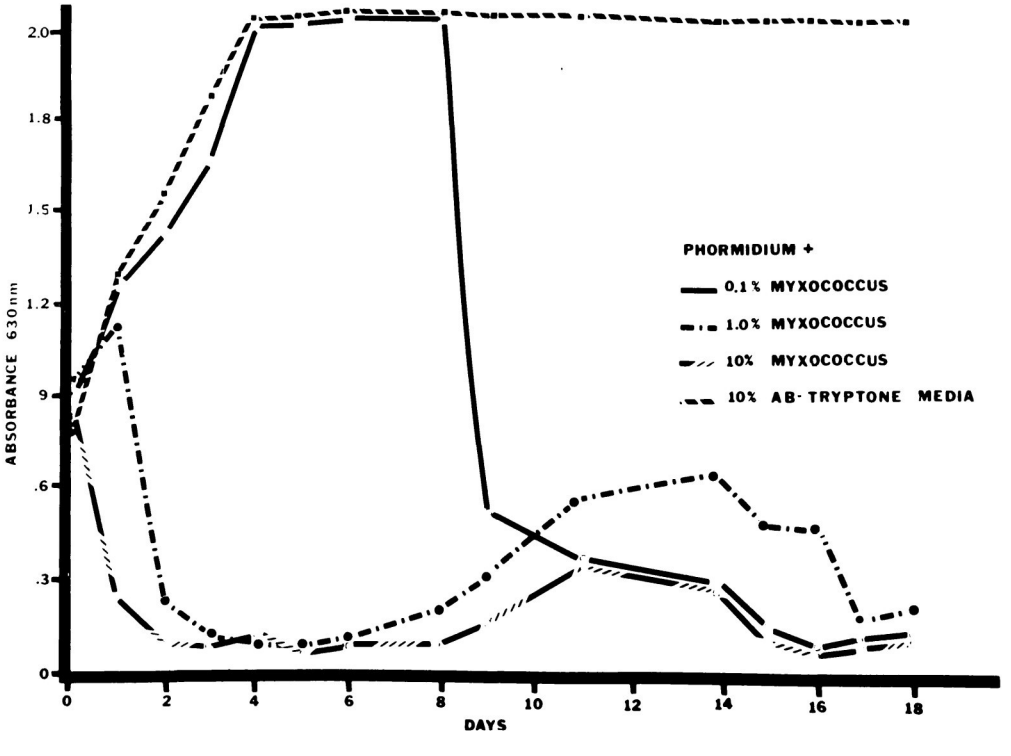
ABSORBANCE 630nm
2.0
1.8
1.5
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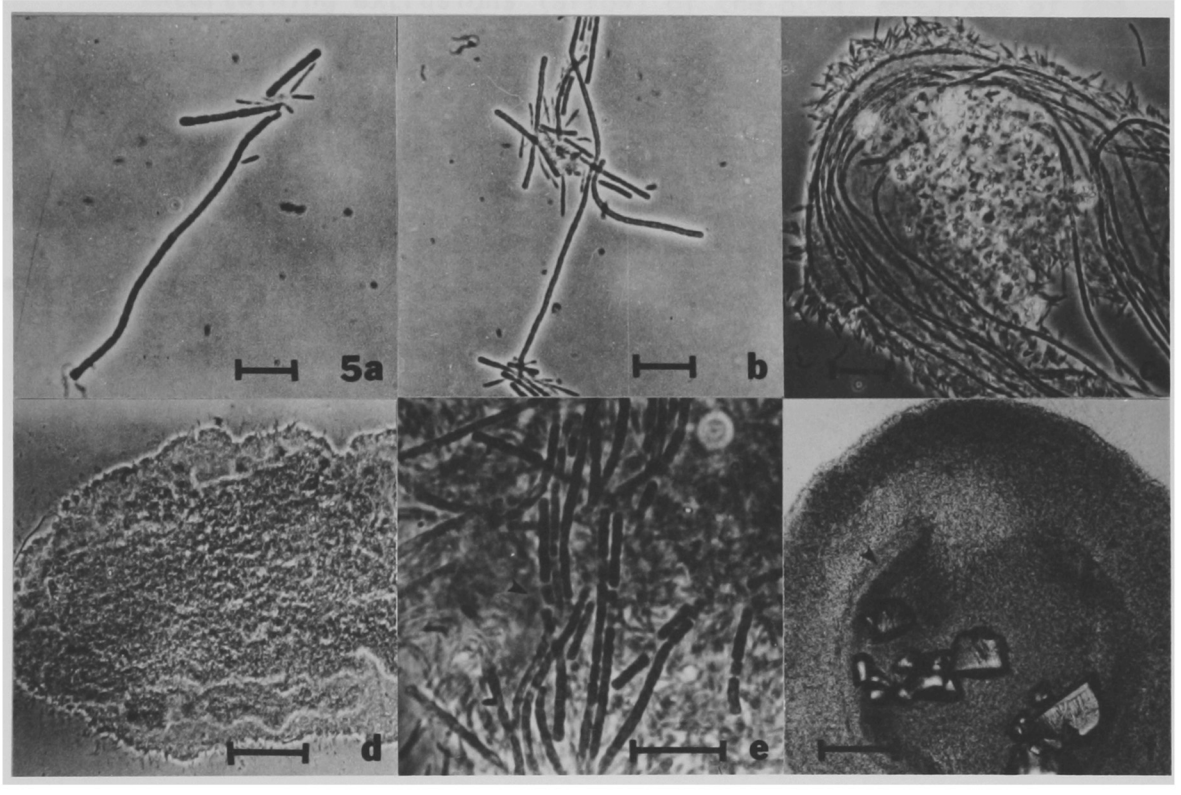
.9
.6
.3
0
0
2
4
6
8
10
12
14
16
18
DAYS
PHORMIDIUM +
0.1% MYXOCOCCUS
1.0% MYXOCOCCUS
10% MYXOCOCCUS
10% AB-TRYPTONE MEDIA

4

PLATE TWO

Fig. 5. Development of M. xanthus PC02 colonies in the presence of 10^7 P. luridum cells/ml (a, b) 17 h interaction, 1% myxococcal inoculum. Note microfloccula formation. Cyanobacterial lysis is indicated by arrow. Bars equal 10 μm. (c) One h interaction, 50% myxococcal inoculum. Bar equals 10 μm. (d) Ninety-six h interaction, 1% myxococcal inoculum. The boundary between the myxococcal periphery and the cyanobacterial core is marked by arrows. Bar equals 100 μm. (e) The core region of the colony shown in (d). Bar equals 10 μm. (f) Six-day interaction, 1% inoculum. The spherule was placed under a coverslip and gently compressed to reveal the internal morphology. Note the large crystals commonly observed in the core of such colonies. Bar equals 50 μm.


5a
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d
e

PLATE THREE

Fig. 6. SEM of a mature colony of axenic M. xanthus PCO2 grown for 5 days in 0.2% tryptone in AB. Bar equals 100 μm.

Fig. 7. An intermediate magnification of the surface convolutions on the spherule shown in Fig. 6. Bar equals 10 μm.

Fig. 8. A high magnification of the spherule shown in Fig. 6. Note the intertwining extrusions (arrow) of the outer membrane of the myxococci. Bar equals 1 μm.

Fig. 9. A TEM thin section through one of the outer membrane extrusions. Bar equals 0.05 μm.

Fig. 10. SEM of an M. xanthus PCO2 colony grown axenically in ABT for 10 days. Note the spiked protrusions on the surface of the colony. Bar equals 100 μm.

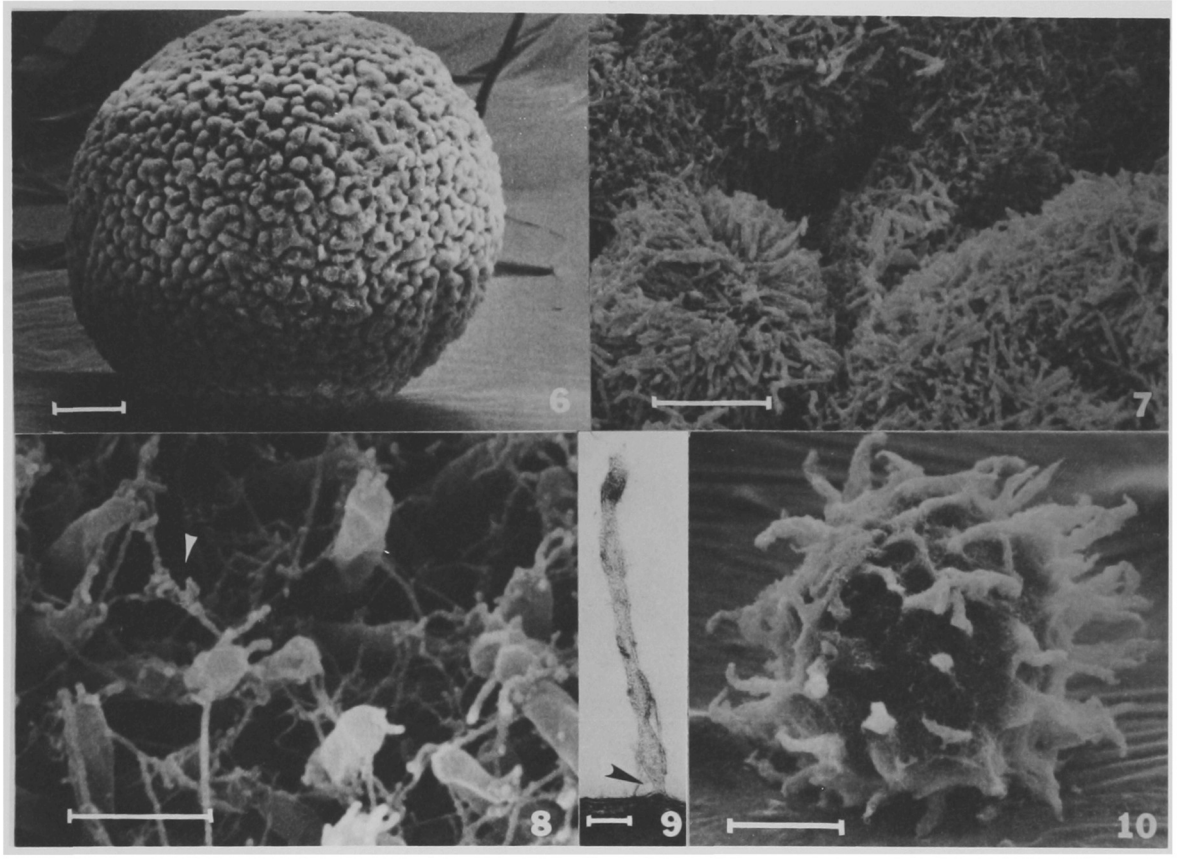
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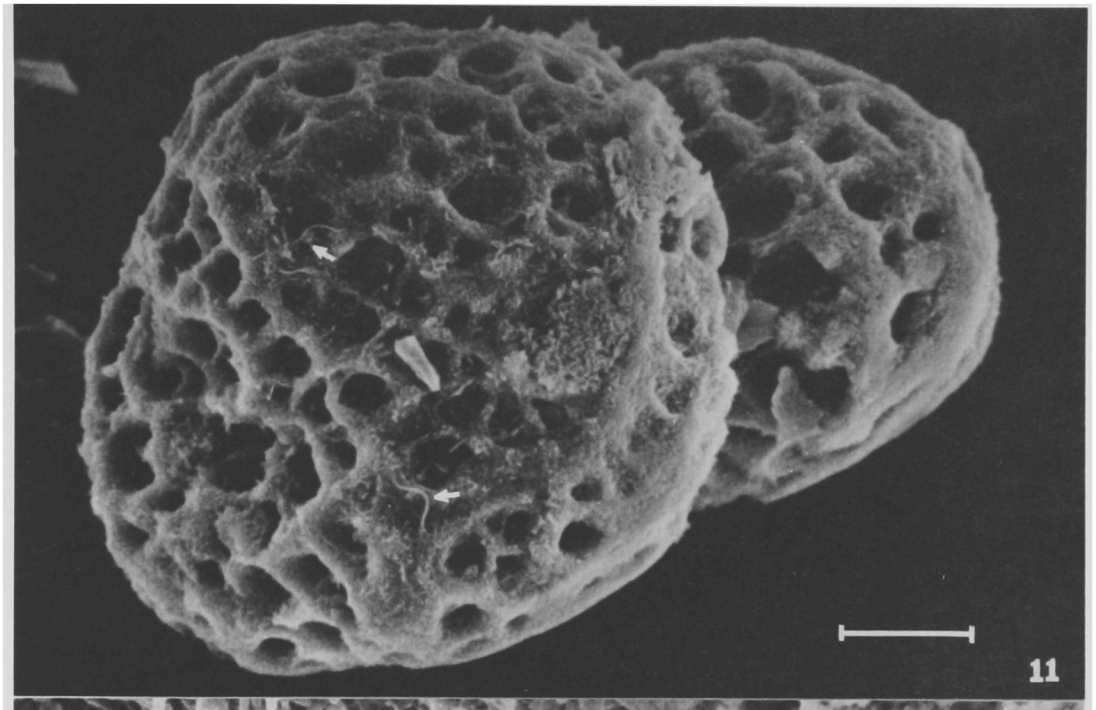
PLATE FOUR

Fig. 11 SEM of two mature colonies of *M. xanthus* PCO2 and *P luridum* taken after 5 days of interactive culture. Arrows indicate the ensnared filamentous cyanobacteria. Bar equals 50 μm.

Fig. 12. A higher magnification SEM of the surface of the spherule in Fig. 9. Note the presence of myxospores (arrows) along the edges of the outer layers of the spherule. Bar equals 5 μm.



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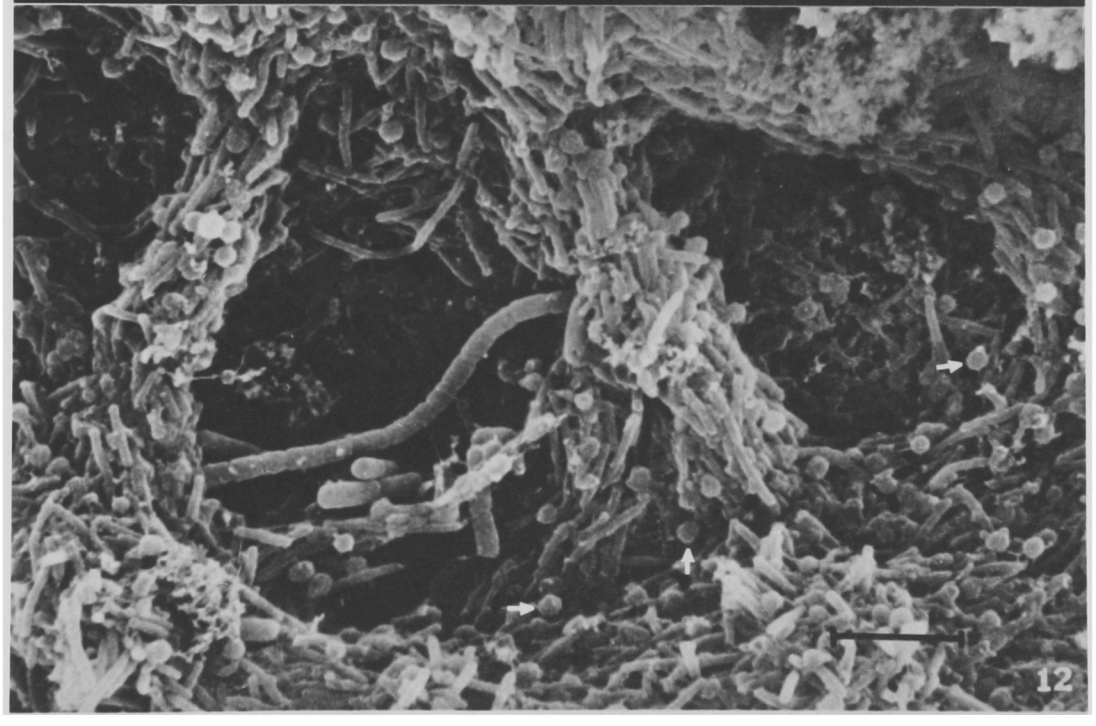

12

PLATE FIVE

Fig. 13. TEM thin section through the outer region of a myxococcal colony formed during 24h incubation with 10^7 P. luridum cells/ml Bar equals 1.0 μm.

Fig. 14. TEM thin section through the outer region of the core of the myxococcal colony. Arrows indicate extrusions from the cell wall of the myxococcal cells. Bar equals 1.0 μm.

Fig. 15. TEM thin section of the central core of a 24 h colony as shown in Figs. 13 and 14. Bar equals 1.0 μm.

Fig. 16. A thin section from the core of a 5-day old colony. Bar equals 0.8 μm.

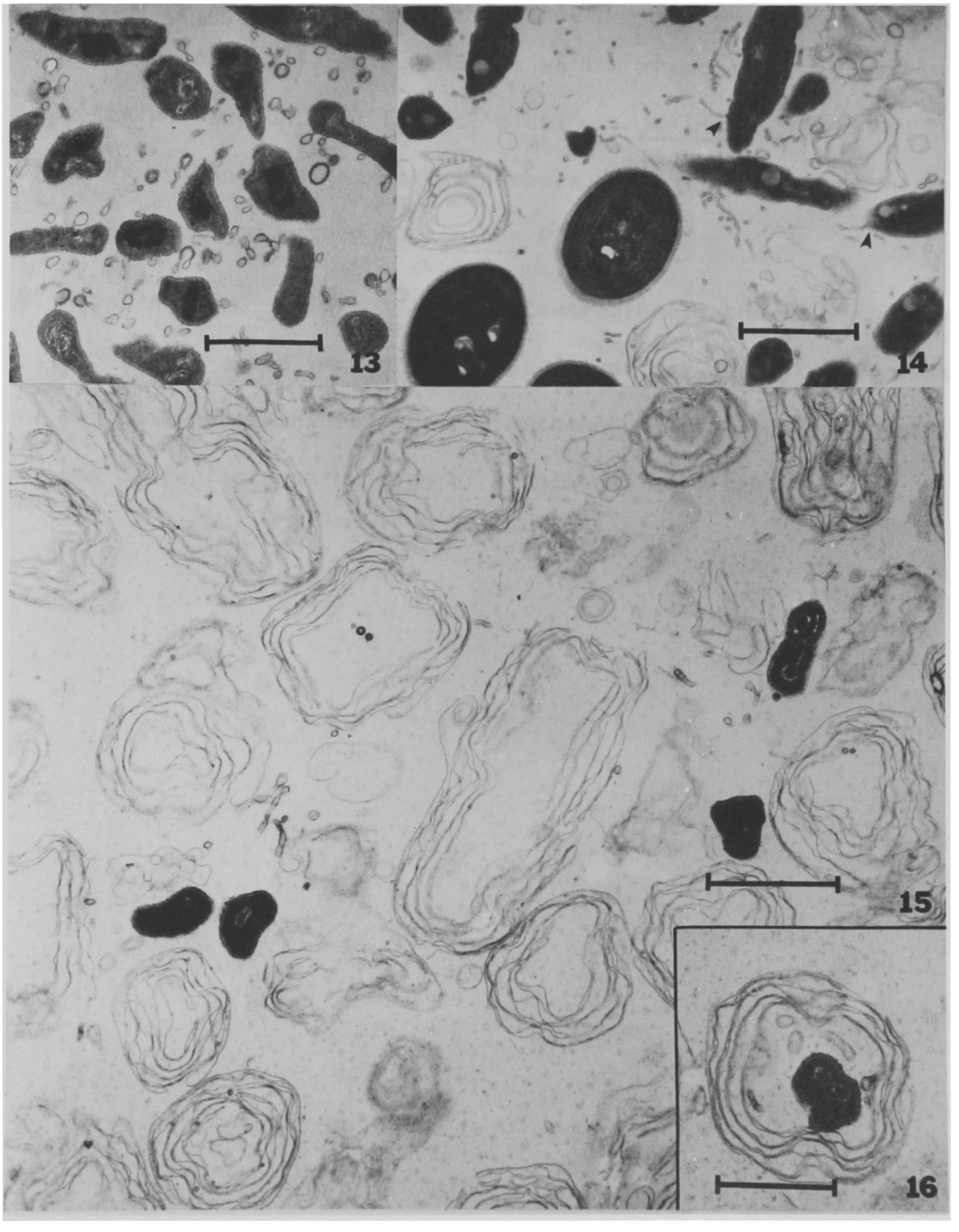
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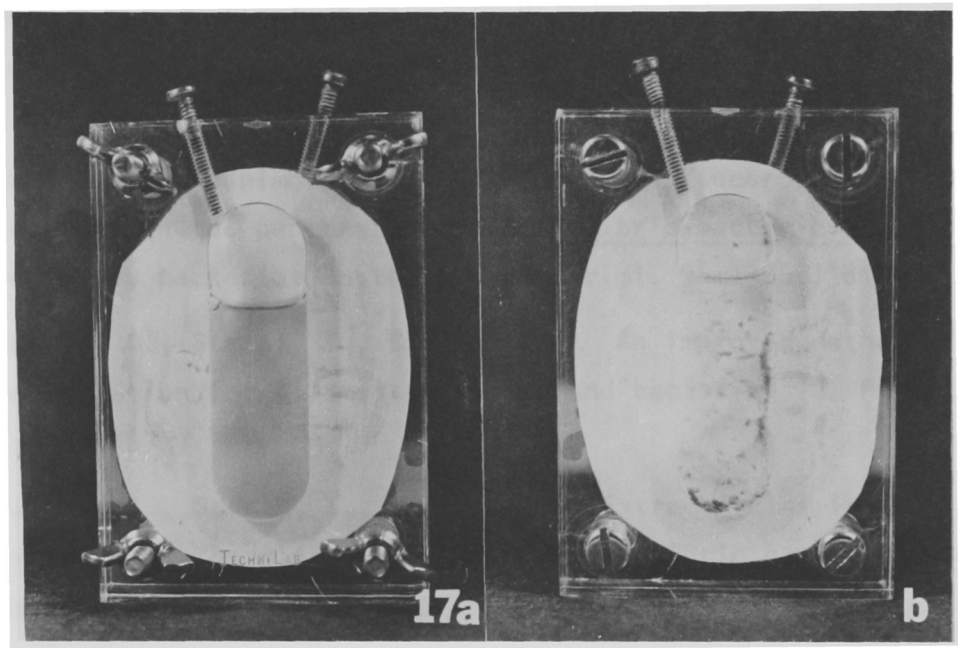
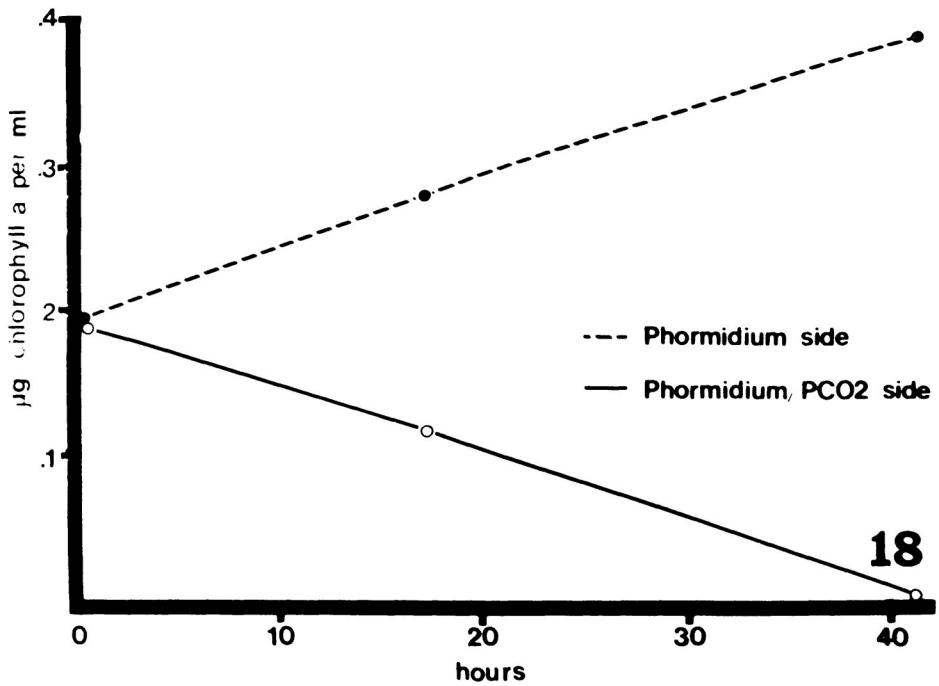
PLATE SIX

Fig. 17. TechniLab plastic dual chamber culture apparatus employing a 0.2 μm pore Millipore membrane as the chamber barrier after 48 h interaction. a) Control side: P. luridum in AB only; b) experimental side: equal numbers (10^7/ml) of P. luridum and washed M. xanthus PCO2.

Fig. 18. Measurement of chlorophyll a concentration in the dual chamber experiment shown in Fig. 17. Special effort was taken to ensure that all specimens for acetone-methanol extraction contained the appropriate percentage of myxococcal colonies.


17a
b


.4
.3
2
.1
µg chlorophyll a per ml
Phormidium side
Phormidium, PCO2 side
18
0
10
20
30
40
hours

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THE UTILIZATION OF BACTERIA IN MANAGING CYANOBACTERIAL POPULATIONS: A REVIEW AND UPDATE

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Introduction

This review will examine a series of papers which have utilized a blend of bacterial physiology and cytology to examine and solve a problem in applied ecology, i.e., how to utilize bacterial populations to control the growth of different cyanobacterial species in natural aquatic habitats. As the earlier papers presented at this conference have amply demonstrated, cyanobacterial problems dominate in many of the lakes and reservoirs in the United States. As nitrates and phosphates accumulate from soil runoff, the waters become eutrophic and the cyanobacterial populations proliferate rapidly to undesirable levels, causing taste and odor problems, poor aesthetics and occasionally toxic gastroenteritis if ingested by man or animals. Most significantly, as these cyanobacterial populations die and decay, the dissolved oxygen in the water is depleted, leading to a total disruption of the desired aquatic ecosystem balance. The rationale of using bacterial biological control agents is to prevent the accumulation of cyanobacteria to noxious levels by applying an interspecies antagonism, i.e., by either inhibiting the growth of or lysing the cells of the cyanobacterial pests. This is amplified by a quotation from Huffaker and Messenger's (1976) book entitled Biological Control: "If we are to reverse the trend toward an ever intensified overloading of the environment with polluting and highly toxic pesticides, we must show that biological control, combined with restricted usage of selective chemicals and other integrative measures can, in fact, solve many of our pest problems without resort to disturbing and polluting chemicals"

The Nature of the Prey

If one is to devise a strategy to effectively antagonize a group of organisms it is important to consider the basic properties of these organisms, as well as to examine specific characteristics of the organisms that would provide benefit to a predator. The cyanobacteria occupy a unique phylogenic and evolutionary niche in nature. Until recently (Buchanan and Gibbons, 1974) these organisms were considered algae and allied closer to the plant kingdom. Their properties are well documented (Fogg et al., 1973) and their procaryotic structure is well established (Lang, 1968), bringing them closer to the bacteria in structure and function. This is important to the philosophy of attacking them in aquatic ecosystems. As outlined in Table I, there are many properties of these cyanobacteria that directly affect the feasibility of bacteria being successful cyanobacterial predators. Their autotrophic metabolism allows these cyanobacteria to convert inorganic nutrients to organic cell constituents. The fact that many cyanobacterial species are abundant in eutrophic waters makes them one of the primary sources of heterotrophic nutrient present in fresh water ecosystems. Unfortunately, for most aquatic bacterial species, this nutrient source is simply unavailable. A successful strategy of an effective predator would be to utilize this common nutrient source to its advantage. The cyanobacterial properties of aerobic growth in surface waters while secreting oxygen and carbohydrate provide both a natural attraction for bacteria and a suitable environment for an oxygen utilizing predator to operate. Their common filamentous property, although providing problems to the investigator studying cellular kinetics, provides a microbial parasite a mechanism with which to engangle the prey. The flocculent clumping that results provides an enormous increase in surface area for continued

collection of suspended cyanobacteria. The procaryotic characteristics of a peptidoglycan cell wall layer (Lang, 1968) offers potential cyanobacterial vulnerability to bacterial cell wall lytic exoenzymes and antibiotics. This character will be extensively developed later in this presentation. Finally, the observation that given considerable stress (Burnham, et al., 1976; 1977) cyanobacteria are capable of lysing under the primary influence of their own enzymes - a property which only enhances the production of available heterotrophic nutrient upon cyanobacterial dissolution.

Cyanobacterial/Bacterial Relationships

Although the interactions between bacteria and cyanobacteria involve symbiotic, commensal, neutral or antagonistic relationships. There have been serious efforts to examine the requirements of either partner for interaction and the resulting nutrient and gaseous exchange between the species. The dominant relationship appears one of symbiotism (Lange, 1970) and with the carbonaceous and nitrogenous excretions of the cyanobacteria (Fogg, 1952) being assimilated by the bacteria, and the bacterial-produced carbon dioxide resulting in accelerated cyanobacterial photosynthesis (Lange, 1971) This relationship was elegantly illustrated in an electron microscopic study by Pearl (1976) of bacteria colonizing the nitrogen fixing heterocysts of Anabaena and Aphanizomenon species. Because atmospheric CO_2 probably becomes limiting during intensive photosynthesis, the CO_2 producing role of bacteria may be certainly beneficial to the rapid growth of the cyanobacteria. This relationship was shown by Lange (1971) to be enhanced with CO_2 production by the symbiotic bacteria was increased by adding various organic substrates and could be mimicked by supplying additional CO_2 to the cyanobacterial culture.

This nutrient exchange between symbiotic or commensal bacteria and cyanobacteria only enhances the concept that cyanobacterial organic compounds could serve as a major nutrient for a bacterial prey species.

Although symbiotism is common, non-specific antagonism also occurs. Fitzgerald (1969) showed that bacteria-containing sewage effluents would support the growth of the green alga Chlorella but would not allow the growth of the cyanobacterium Microcystis aeruginosa. When the bacteria were removed by autoclaving or filtration the M. aeruginosa were able to thrive. Gunnison and Alexander (1975) in a study examining why certain algae could be naturally degraded by microbial enzymes showed that the peptidoglycan component of cyanobacteria (bluegreen algae) provides the weak link in these organisms' armor against microbial lysis. Fallon and Brock (1979) enlarged on this concept of microbial lysis of algae by examining the decomposition and mineralization of cyanobacteria in a lake in Wisconsin. They concluded that the bacteria responsible for cyanobacterial degradation depended upon the products of that degradation for all of their nutritional needs. Although these authors report a lytic bacterial level of 10^3 cells per ml of tested lake water, they did not identify the bacterial decomposer species. The remainder of this review will examine specific antagonistic relationships between bacteria and cyanobacteria.

Bacterial Antagonism for Cyanobacteria

Table 2 provides a list of the bacterial systems that are capable of causing the lysis of cyanobacterial populations. I should point out that other microorganisms, specifically various protozoa, fungi and cyanophage, are also capable of lysing cyanobacteria but these will not be considered in this presentation. I have discussed many of the lytic bacteria in a previous review (Burnham, 1975), so I will highlight only

a few in this presentation. Bacterial lytic secretions without direct cell-to-cell interaction explains the mechanism of lysis for most of the lytic genera listed in Table 2. Actinomycetes, Streptomyces, Bacillus, Pseudomonas, Cellvibrio and Bdellovibrio cell-free culture supernatant preparations have been reported to contain cyanobacterial lytic substances, either exoenzymes or antibiotics. Because the cyanobacterial antagonism that results from these interactions depends upon the concentration of these lytic substances in the environment, I do not believe they offer potential as practical control agents. My own earlier efforts utilizing Bdellovibrio bacteriovorus (Burnham, 1975; Burnham, 1976; Burnham et al., 1976; Burnham and Sun, 1977; Burnham, 1977) evinced an interesting inhibitor of cyanobacterial photosynthesis of low molecular weight that triggered an autolytic dissolution of the photosynthetic lamellae. Such a photosynthetic toxin was appealing as a control agent but my laboratory has not been successful in stimulating the production of this inhibitor without large additions of protein to the bdellovibrio culture. Until we can demonstrate the production of this inhibition without direct substrate addition we do not believe the bdellovibrio system can be of significance in environmental cyanobacterial control.

The lysis of cyanobacteria by the myxobacteria in my opinion offers the best potential at the present time for a successful biological control agent for unwanted cyanobacteria. Even since Shilo, 1967 showed that myxobacteria were capable of lysing various species of cyanobacteria evidence has been accumulating which only heightens this potential.

Shilo's discovery simply expanded upon the knowledge that myxobacteria are one of the most potent bacteriolytic microbial organisms known. Early data (Beebe, 1941) indicates that these organisms were readily able to lyse living host bacterial populations. This lytic characteristic is due to its ecological niche as a soil bacterium and in addition to

production of enzymes during its life cycle processes of fruiting body and cyst formation (Kottel and White, 1974)

Wu et al. (1968) indicated that an unidentified myxobacterium was capable of lysing in a liquid culture strain of Lyngbya and five other bluegreen species. The authors indicated that lysis was associated with a slow "clumpy" growth of the myxobacterium and the production of a lysin.

Stewart and Brown (1969) isolated a Cytophaga which formed plaques on both green and bluegreen algae. These authors indicated that the lysis of the algae to be extracellular, but the exact cause of lysis was not described.

Shilo (1970) isolated a myxobacter (designated FP-1) that lysed viable vegetative cells of many unicellular and filamentous bluegreen algae. Lysis in liquid cultures was prevented when the algal cultures were shaken. Light microscopy demonstrated that algal lysis only occurred upon polar attachment of the myxobacter to the algal cell. Detection of excreted lytic enzymes was unsuccessful, suggesting that the lytic enzymes may be bound to the surface of the myxobacter.

Five algicidal non-fruiting myxobacteria were described by Stewart and Brown (1971) to have a uniformly high G+C ratio of approximately 70 mole percent. All of these organisms were effective in lysing algae but none of these bacteria were capable of forming microcysts, a feature which distinguishes them from the Myxococcus PC02 isolate. Myxobacter has been a general name for any bacterium falling within two orders, Myxobacteriales and Cytophagales. Using the criteria described by Stewart and Brown (1971) their isolates would be grouped as members of the Cytophaga genus by the 9th edition of Bergeys Manual (Buchanan and Gibbons, 1974)

Daft and Stewart (1971) described four myxobacters that could lyse 40 strains of bluegreen algae. Again cell contact appeared to be necessary for lysis to occur. The authors suggested that one bacterium can initiate lysis of the algae. Although lysis took from 2 to 7 days photosynthesis was inhibited about 85% after 10 hours. Daft and Stewart (1971) indicate that these myxobacteria may be important in regulating algal development in nature.

The structural basis for algal lysis by the Myxobacterium CP-1 was described by Daft and Stewart (1973). The primary ultrastructural effect was the dissolution of the L2 or mucopeptide layer in the cell wall of the bluegreen algae tested. Large intrathylakoidal spaces were seen to form; however, the membranes themselves seemed very resistant to myxobacter CP-1 disruption. This pathology of the photosynthetic system is very similar to that described for bdellovibrio interaction with Phormidium luridum (Burnham and Sun, 1977) Daft and Stewart (1973) point out that the concentration of bacteria employed in these structural studies were far in excess of those encountered in nature. Generally, a 1:1 proportion of bacteria with algae were employed in their studies.

The physiologic conditions under which algal lysis by various myxobacteria occurred was reported by Daft et al. (1975) The lytic bacteria were all strict aerobes. Lysis increases as the PO_2 was increased to 45%. Higher levels were inhibitory. The pH optima for lysis was within the range of 7.0 to 9.0 for all strains of myxobacteria tested.

Lysis was not reported at 37 C for strain CP-1. Daft et al. (1975) suggest that optimum lysis in the field should be expected in the summer months in shallow water as the pH will also be quite suitable. The number of myxobacteria per ml of lake water ranged from 4 to 400. These authors showed that in surveying 8 bodies of water in Scotland (5 lakes,

2 reservoirs and 1 sewage plant) there was always a direct statistical correlation between chlorophyll a concentration in the water and the abundance of these lytic bacteria.

The report by Burchard (1975) of colonical spherule formation by M. xanthus in axenic culture provided significance to the feasibility of myxococci as a biological control agent. It conclusively demonstrated that the myxococci possessed a capability for orderly aggregation in liquid environments. This was important in view of their earlier established aggregative properties on semi-solid media (Dworkin, 1973)

Myxococcal cyanobacterial entrapment and lysis

A Myxococcus xanthus designated PC02 was isolated in Port Clinton, Ohio from a roadside ditch that evinced excellent lysis on agar grown lawns of cyanobacteria. When the organism was tested in aqueous cultures of Phormidium luridum, a filamentous cyanobacterium, I found that the cyanobacteria became clumped, overgrown by the myxococci and finally lysed. Although much of the research has been recently reported (Burnham et al., 1979; 1980a; 1980b), I would like to review the major characteristics of this lytic system in this presentation.

The antagonism of M. xanthus PC02 toward the cyanobacterium P. luridum is clearly illustrated in Figs 1 and 2. Figure 1 shows that upon repeated transfer in an autotrophic medium the cyanobacterium alone was always capable of multiplying sufficiently to prevent being diluted out. The P. luridum plus myxococci under similar autotrophic conditions, however, was not capable of multiplying and the 5% transfers rapidly diluted out the cyanobacteria to undetectable levels. Figure 2 shows that upon extended coincubation the myxococci can effectively lyse large numbers of cyanobacteria and maintain the environment at a reasonably low level of cyanobacterial cells per ml. Some cycling of the culture is periodically

observed. The P luridum are able to multiply by several logs; however, this increased growth is again soon lysed by the residual myxococci. This cycling demonstrates the inability of the M. xanthus PC02 system to completely remove all cyanobacteria from the environment. Significantly, I have found that lysis of a culture can be accomplished with predator to prey ratios of 1:100,000. This is significant in view of the need to be able to use low inocula in natural ecosystems if practical usage is to ensue.

Figure 3 shows the ability of the M. xanthus PC02 strain to lyse P. luridum on agar lawns. The photograph further demonstrates the spreading or gliding motility that the myxococci possesses. These myxococci are normally maintained on lawns of prey cyanobacteria as it ensures that the predatory ability will be retained and even increased as a result of selection of the cells most rapidly clearing the cyanobacteria.

The initial clumping that occurs shortly following the addition of the myxococci to a cyanobacterial culture develops over 12 to 24 hrs into very distinct colonial spherules. With low magnification phase contrast microscopy (Fig. 4) it can be observed that the spherule is made up of an outer region and a core. If the photograph was in color it would be apparent that the outer region was yellowish while the core was a dense green. The spherule is able to concentrate the cyanobacteria into the central regions of the spherule as demonstrated by thin sectioning the spherules and examining the core by transmission electron microscopy (Burnham et al., 1979; 1980b). Mature colonial spherules often reach a diameter between 1 to 5 mm.

When axenic spherules of myxococci alone are examined using parafin embedment and light microscopy the separation of core and myxococcal growth at the surfaces of the spherule is very clear (Figure 5) High

magnification phase contrast microscopy of a young spherule (Figure 6) shows the large numbers of myxococci surrounding an entrapped P. luridum filament which is in the process of being degraded by myxococcal enzymes. Because the spherules increase in size with age and addition of cyanobacterial prey, and because the entire system operates under autotrophic conditions, the cyanobacteria must be serving as nutrient for myxococcal growth.

Closer examination of the surface of the colonial spherule by scanning electron microscopy (Figure 7) shows a distinct orientation of the myxococcal cells with several of the larger cyanobacterial filaments protruding from the spherule surface. Dworkin (1973) and Kaiser et al. (1979) have shown that the myxococci are aggregative bacteria that often swarm over a surface in the process of organizing the colony for production of fruiting structures. I postulate that myxococcal swarming is both the process responsible for the parallel orientation of the bacteria at the surface of the spherule and the primary mechanism by which the myxococci concentrate the cyanobacteria in the spherule core. By constantly gliding over the outer regions of the spherule the myxococci cover the cyanobacteria and by continual myxococcal shifting the cyanobacteria are gradually deposited in the core.

Figure 8 shows the stringy protrusions of lipopolysaccharide (Burnham et al., 1980b) that tie the spherule together. These plus the fimbriae that have been demonstrated by Dobson and McCurdy (1979) also appear to serve as tentacles assisting in the entrapment of cyanobacteria from the surrounding medium.

Finally, I believe the lysis of the cyanobacterial cells within the spherule core occurs because of the well described exoenzymes produced by the myxococci.

Myxobacterial Lytic Enzymes

In studying the myxobacter strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell wall lytic activity. These two functions were inseparable upon purification.

Hart and Zahler (1966) studied a lysin produced by M. xanthus FBa. Purification yielded two distinct enzymes, a lysozyme and a protease. The lysozyme was very effective in lysing cell walls of various microorganisms.

Further purification of M. xanthus FB bacteriolytic enzyme was described by Sudo and Dworkin (1972) BY gel separation techniques an amidase, a glucosaminidase, two proteases with amidase activity and a peptidase active against cell wall peptides were isolated. These are all individually capable of bacteriolytic activity and collectively they appear to indicate why the Myxococcus and its related genera are such potent antimicrobial parasites.

Haska (1974) purified the peptidase produced by a related species, M. virescens and identified it to be a D-alanyl-N lysine endopeptidase, an enzyme that would cause the destruction of the L2 (mucopeptide) layer as observed by Daft and Stewart (1973)

An alternative mechanism for the lysis of algal species could relate to the autolytic system that has been described for M. xanthus FB (Kottel and White, 1974). This enzyme system is induced during myxocyst formation. The release of these enzymes which appear to result in the dissolution of cell walls could lyse walls of sensitive cyanobacterial strains. Wireman and Dworkin (1977) further characterized this autolysis in terms of its sequence in the morphogenic events leading to myxocyst development. The formation of the myxocyst appears to be dependent on the cell-free concentration of cytoplasmic constituents from lysed myxococci. This necessity for lysis of a certain percentage of the total myxococcal

population provides the rationale for the autolytic mechanism.

Myxococcus xanthus strains have been shown to also produce an antibiotic active against both Gram-positive and Gram-negative bacteria. The antibiotic appears to be bacteriocidal. Escherichia coli B cells when exposed to this myxococcal antibiotic for 60 min all showed lysis (Rosenberg et al., 1973) Vaks et al. (1974) characterized the antibiotic to be active only against growing cells.

Finally, it has been reported recently that certain proteases are bound to the extracellular slime found associated with M. virescens B2 (Gnosspelius, 1978). The author suggests that these enzymes could play an important role in denaturing protein components from microbial prey cells lysed by myxobacterial activities.

Summary and Recommendations

The M. xanthus colonial spherules, to the extent they have been tested in my laboratory, offer excellent potential as microbial control agents for cyanobacteria. The specific advantages of these lytic mechanisms are listed in Table 3. Heading the list is the primary reason why I am enthusiastic about this lytic system, i.e., it is capable of fuctioning in the total absence of heterotrophic nutrient other than aqueous cyanobacteria. This is in complete contrast to the Bdellovibrio bacteriovorus system previously described (Burnham et al., 1976) which requires a high concentration of exogenous protein in order to function. Also distinctive is the ability to carry out cyanobacterial lysis at high agitation rates. This is due to the confined nature of the lytic system and the ability of the M. xanthus to swarm over the cyanobacteria, thereby moving them to the core regions. The encystment ability of the M. xanthus species allows predator survival in periods of adversity such as winter or reduced availability of cyanobacterial hosts. Although Daft et al. (1975)

found a significant reduction in myxobacterial predator counts in the winter versus summer in Scottish waters, they did at least document the survival of the predator species. My laboratory has successfully tested 7 strains of cyanobacteria for prey status. This plus the results of Daft and Stewart (1971) indicating that the major cyanobacterial bloom producers (ex. Aphanizomenon flosaquae; Microcystis aeruginosa; Anabaena circinalis; A. spiroides and Caelosphaerium) were all lysed by myxobacteria further heighten the control potential for this type of bacterium. Following continued monitoring of various environmental parameters and larger scale testing in tanks I hope to develop a testing program utilizing controlled pond situations.

The various viewpoints presented at this conference suggest a series of recommendations with regard to microbiological control system development: (a) The data produced in my own laboratory and that from the other research cited in the review, particularly that of Daft et al., 1975, indicates microbial control has a potential value to the management of cyanobacteria in lakes and reservoirs. (b) With the increasing costs associated with lakes management that this conference has demonstrated it appears that the favorable cost/benefit ration that could be provided by biological control only increases the need for successful development of a microbial control system; (c) With the need for an effective microbiological control system evident, funding agencies should approach various disciplines, not only microbiology, with "request for proposals" to develop more innovative ideas in this field. One significant accomplishment of the conference was to bring together experts from many disciplines and the resultant exchange was very stimulating. Such an approach could work in funding new research. (d) A funding agency might utilize the multidisciplinary approach to review projects as well. If an idea appeared collectively successful to a multidisciplinary lake or water management group it could be given increased priority over traditional reviews. (e) Research in the field of microbial control needs to be

simultaneously (1) developed in order to understand the nature of the biochemical structural interactions that occur between predator and prey; and (2) evaluated in increasingly complex systems which progressively mimic the natural ecosystems that are targeted. (f) The funding mechanism should include sufficient commitment to provide testing in controlled natural ecosystems such as test pond environments. (g) Successful programs should be integrated with other physical and biological techniques into a well-monitored lakes management program.

The establishment of such a program could bring about the improvement in water quality we all desire in a manner consistent with the challenge to avoid further toxification or pollution of the aquatic ecosystem.

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The technical assistance of Susan Collart and Barbara Highison is appreciatively recognized.

TABLE 1

Characteristics of Cyanobacteria Influencing Their Role as Microbial Prey

abundance in eutrophic waters

autotrophic metabolism

aerobic

growth in surface waters

oxygen producers

secrete carbohydrates

filamentous

peptidoglycan cell wall component

autolytic mechanism

heterotrophic nutrient release upon lysis

TABLE 2

Bacterial Lytic Systems for Cyanobacteria

Lytic Bacterial Genera	References
Actinomyces	Safferman and Morris, 1962 Sladekova and Sladek, 1968
Streptomyces	Gunnison and Alexander, 1975
Bacillus	Reim et al., 1974
Pseudomonas	Mitchell, 1972
Cellvibrio	Granhall and Berg, 1972
Bdellovibrio	Burnham et al., 1976
Myxobacteria	Stewart and Brown, 1971; Wu et al., 1968; Daft and Stewart, 1971;1973; Daft, McCord and Stewart, 1975; Shilo, 1970
Cytophaga	Stewart and Brown, 1969
Myxococcus	Burnham, 1979; 1980 a, b

TABLE 3

Advantages of the Myxococcus xanthus lytic spherules as a microbial biological system

1. effective in autotrophic environment
2. utilization of dominant microorganisms as nutrient in eutrophic aquatic systems
3. low inoculum of predator effective
4. independent of environmental agitation
5. non-specific host requirement
6. effective host entrapment mechanism
7. lytic system contained and segregated
8. multicomponent nature of lytic system
9. encystment ability of predator
10. predator survival in hostile environments

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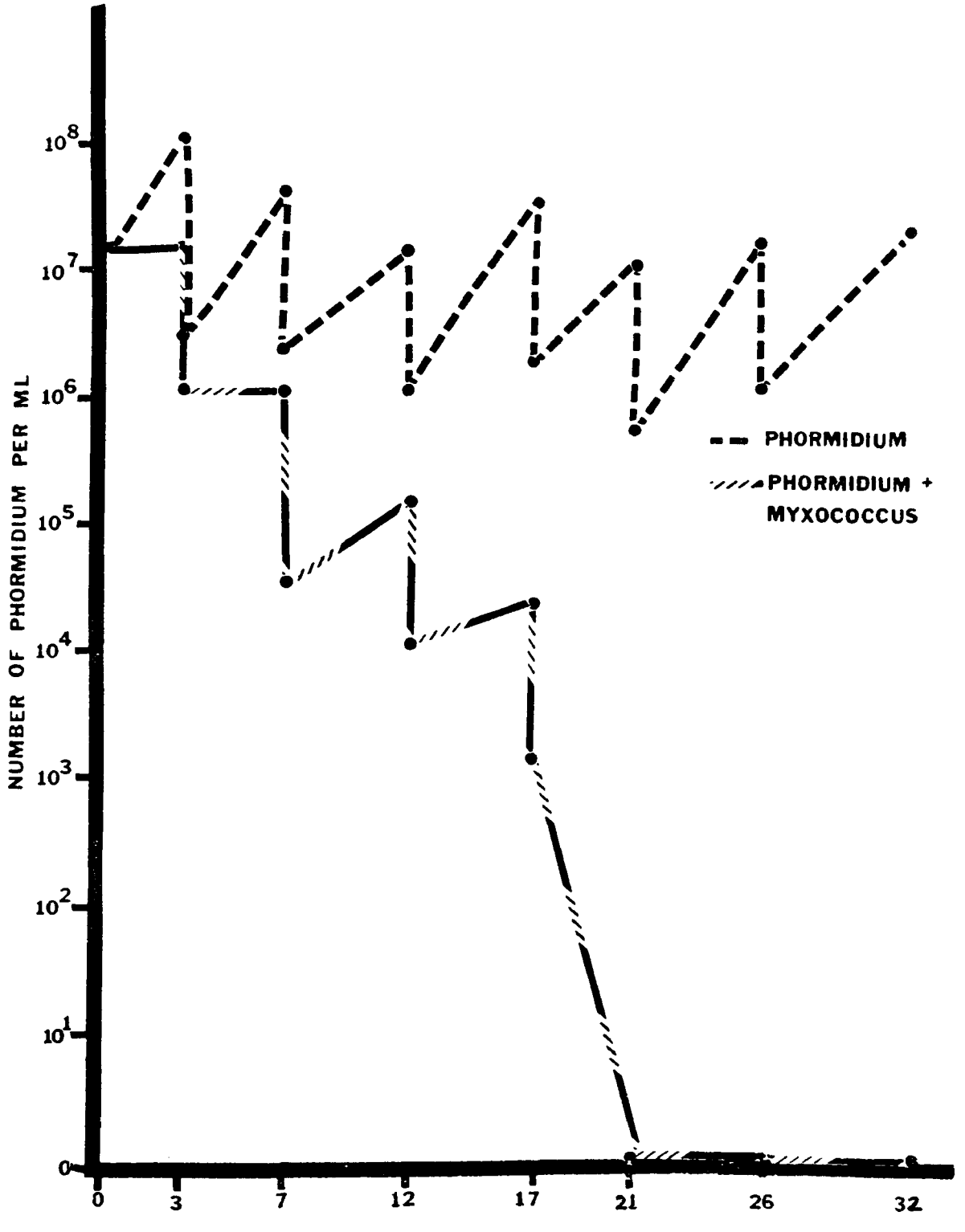
Legends for Figures

Figure 1. This graph illustrates the result of repeated 5 percent serial transfers (indicated by the vertical lines) on axenic cultures of the cyanobacterium P. luridum and an interactive mixture of washed M. xanthus PC02 ($1x10^6$ cells/ml) and P. luridum ($2x10^7$ cells/ml) into a fresh flask of algae broth (Difco) As can be seen from the upper dashed lines, when the P. luridum cells alone were transferred, they multiplied back to approximately original levels. When the coincubated microorganisms were transferred the M. xanthus PC02 prevented this multiplication of the P. luridum so that within five transfers the cyanobacteria were not detectable by microscopic counting procedures.

Figure 2. This graph shows the rapid clearing of a cyanobacterial culture of P. luridum by a large inoculum (10%) washed M. xanthus PC02. This curve also illustrates the cyanobacterial cycling that we have repeatedly found. Following initial lysis by the myxococci, the cyanobacteria are able to partially recover over the period of about a week, only to be eventually lysed again by the myxococcal spherules.


NUMBER OF PHORMIDIUM PER ML
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
0
3
7
12
17
21
26
32
PHORMIDIUM
PHORMIDIUM + MYXOCOCCUS

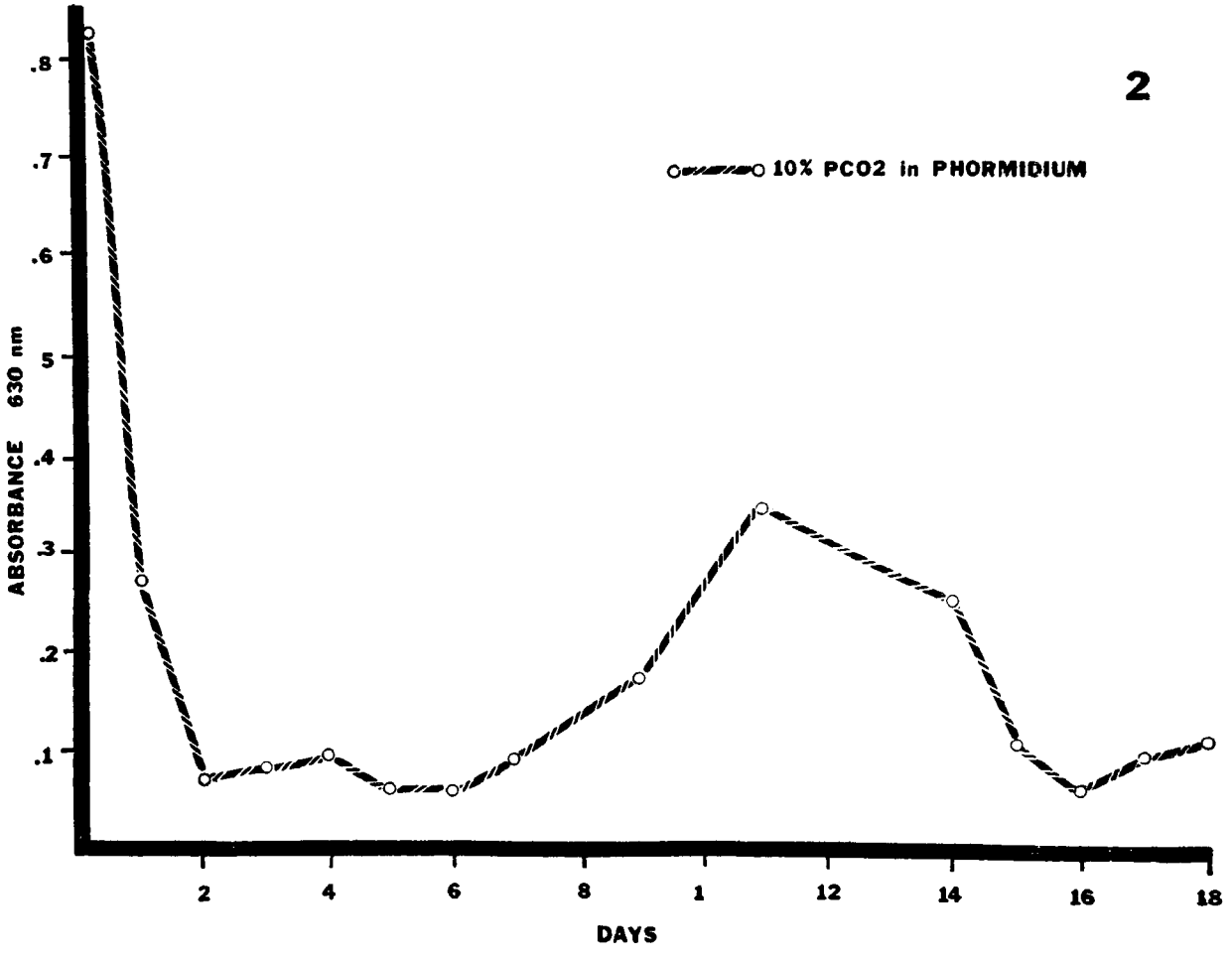

2
10% PCO2 in PHORMIDIUM
ABSORBANCE 630 nm
.8
.7
.6
.5
.4
.3
.2
.1
2
4
6
8
1
12
14
16
18
DAYS

Figure 3. A macrophotograph of an algae agar-containing petri plate upon which a mature lawn of the cyanobacterium, P. luridum (darker region) has been grown. An agar block containing M. xanthus PC02 was placed in the center of the mature lawn. The concentric spreading cyanobacterial lysis caused by the gliding myxococci can be seen in the clear circular zone on the plate. The rectangular zone at the edge of this circle is where an agar block was removed and transferred to another cyanobacterial lawn. This degree of lysis is commonly seen after 5 to 7 days. Magnification: .9X.

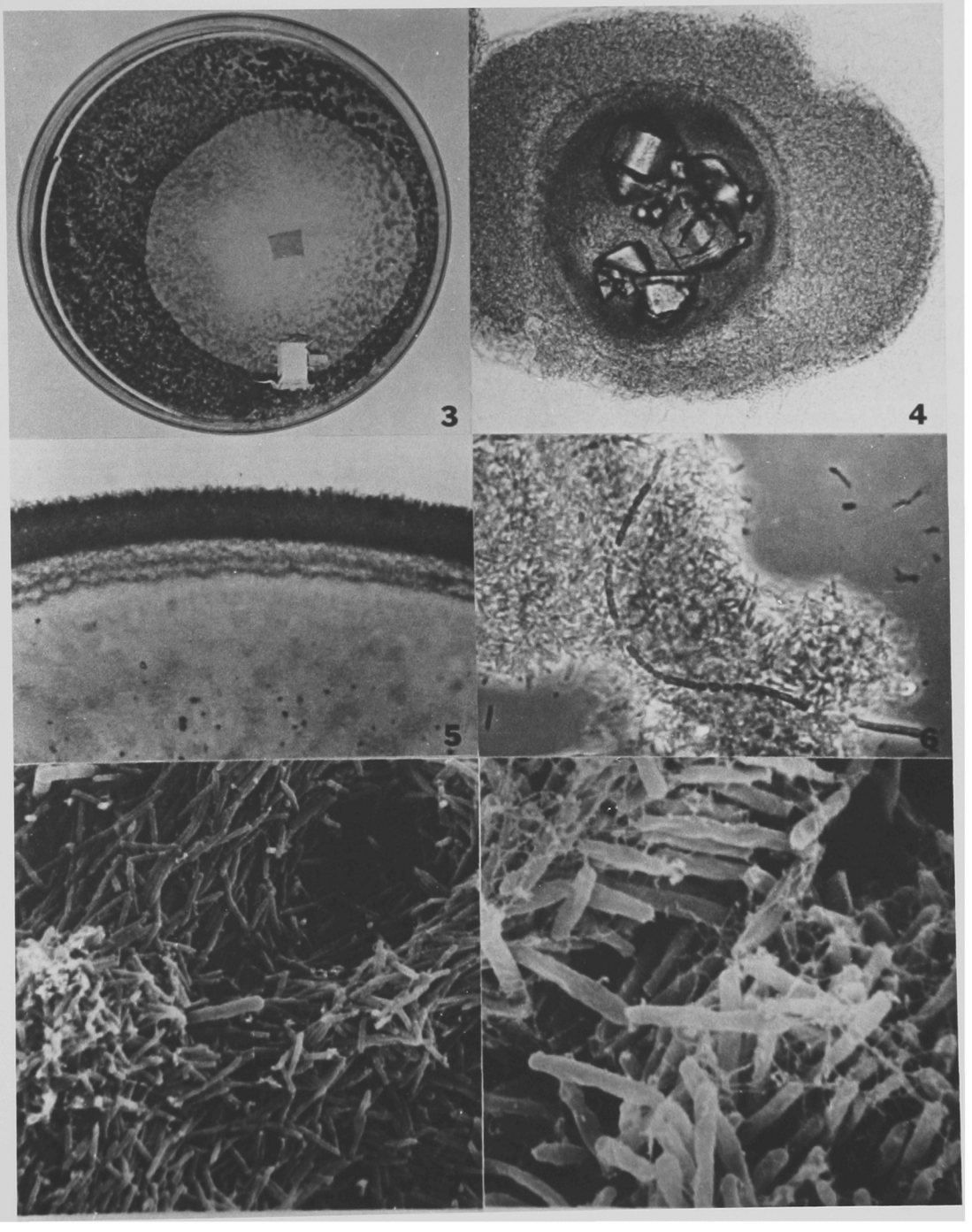
Figure 4. This phase contrast micrograph of a partially compressed M. xanthus PC02 spherule containing the cyanobacterium M. xanthus PC02 in the core (dense) region. Also present in the core are numerous crystals that appear to have a function in the flocculation that occurs in the early stages of spherule formation. At the surface of the spherule numerous myxococcal cells can be seen diffusing away from the spherule as a result of the compression technique. Magnification: 180X.

Figure 5. This bright field macrograph shows a Gram stained parafin embedded spherule of M. xanthus PC02. The outer dense growth of the myxococcal cells is apparent as is the relatively unstructured core (lighter) region. The significance of the intermediate banding by the myxococcal cells has not been determined. Magnification: 315X.

Figure 6. This phase contrast micrograph shows a filament of P. luridum completely surrounded by vegetative cells of M. xanthus PC02. The filament is in the early stages of degradation as evinced by the separation of the filament into individual cells. Magnification: 1,020X.

Figure 7. This scanning electron micrograph shows the parallel orientation of many of the myxococcal cells on the surface of a spherule. The larger filamentous P. luridum can be seen protruding from the surface in several locations. Also noticable are ridges on the surface of the spherule formed by hundreds of the myxococci in parallel orientation. These are postulated to be due to the swarming action of the myxococi over the spherule surface. Magnification: 2650X.

Figure 8. This scanning electron micrograph shows the rod-like M. xanthus PC02 cells joined together by small fiber-like protrusions. Transmission electron microscopy confirmed that these are composed of lipopolysaccharide and are contiguous with the outer membrane of the cell wall of the myxococcal cells. The entanglement of the fibers holds the sphere into shape and suggests a role of the entrapment of the cyanobacteria. Magnification: 7015X.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ABSTRACT FOR SECOND INTERNATIONAL SYMPOSIUM
ON MICROBIAL ECOLOGY

September 7-12, 1980, University of Warwick, Coventry. England

Myxobacterial Entrapment and Lysis of Aquatic Cyanobacteria

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Two Myxococcus strains (PCO2 and BGO2) isolated in Northwest Ohio, USA, show a unique ability to entrap filamentous cyanobacteria within colonial spherules. The cyanobacteria in the center of these densely green spheres undergo degradation with the liberated nutrient being used by the myxococcus for further growth and sphere expansion. Electron microscopy shows the spherule formation and cyanobacterial entrapment to be aided by fimbriae and long lipopolysaccharide extrusions. Serial transfers have demonstrated the long-term survival of the myxococci on degraded cyanobacteria, continual antagonism toward viable cyanobacteria, and the potential of this system as a biological control mechanism.

N 74 Photorespiration During Thermally-and Bacterially-Induced Stress in the Cyanobacterium, Phormidium luridum. J.C. BURNHAM*, G. LOCHER, D.C. SUN, Dept. of Microbiology, Medical College of Ohio, Toledo, Ohio.

Similar 30 min. exposures at 1200 fc light of a 6-day bacteria-free Phormidium luridum culture to either a 10,000 MW fraction of a Bdellovibrio bacteriovorus 15143 culture supernatant or to a 55°C environment yielded photorespiratory levels of -0.44 ul $O_2 \cdot hr^{-1} \cdot ugm\ Chla^{-1}$ and -0.30 ul $O_2 \cdot hr^{-1} \cdot ugm\ Chla^{-1}$, respectively. These levels compare with +1.03 ul $O_2 \cdot hr^{-1} \cdot ugm\ Chla^{-1}$ produced by control P. luridum photosynthesis at 30°C. While dark respiration remained at control levels (-0.09 ul $O_2 \cdot hr^{-1} \cdot ugm\ Chla^{-1}$) upon exposure to these stresses, photorespiration increased oxygen uptake approximately 4-fold. Exposure of the cyanobacterial culture to either the bdellovibrio-produced crude toxin or to 55°C for periods of up to 24 hr continued to yield significant photorespiratory values in spite of the gradual structural degradation of the cyanobacterial filaments. Scanning and transmission electron microscopy of P. luridum during photorespiration revealed specific damage to both the cell wall and photosynthetic membranes. A similar photorespiration was measured when P. luridum was treated with 10 ug/ml of polymyxin B and amphotericin B, but not when either was used singly.

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PHYSIOLOGICAL AND ULTRASTRUCTURAL CHANGES IN *PHORMIDIUM LURIDUM* INDUCED BY EXPOSURE TO BACTERIAL TOXINS AND THERMAL STRESS

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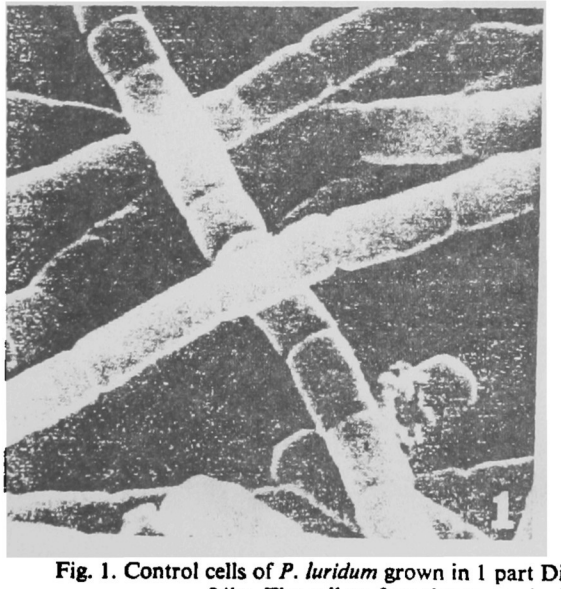
Previous data have shown that *Bdellovibrio bacteriovorus* and its secretions cause metabolic and pathological changes in cells of the cyanobacterium, *P. luridum* (Burnham *et al.*, 1976; 1977a,b). The present study expands the ultrastructural aspects of the interaction and shows that the *bdellovibrio*-produced toxin exhibits similar effects on the cyanobacterial cells as severe heat treatment.

Similar 30min exposures at 1200fc light of a 6-day bacteria-free *Phormidium luridum* culture to either a 10,000 mol. wt fraction of a *Bdellovibrio bacteriovorus* 15143 culture supernatant or to a 55°C environment yielded photorespiratory levels of $-0.44\mu l\ O_2 . hr^{-1} . \mu g\ m\ Chla^{-1}$ and $-0.30\mu l\ O_2 . hr^{-1} . \mu g\ m\ Chla^{-1}$ produced by control *P. luridum* photosynthesis at 30°C. While dark respiration remained at control levels ($-0.09\mu l\ O_2 . hr^{-1} . \mu g\ m\ Chla^{-1}$) upon exposure to these stresses, photorespiration increased oxygen uptake approximately 4-fold. Exposure of the cyanobacterial culture to either the bdellovibrio-produced crude toxin or to 55°C for periods of up to 24hr continued to yield significant photorespiratory values in spite of the gradual structural degradation of the cyanobacterial filaments. Scanning and transmission electron microscopy of *P. luridum* during pnotorespiration revealed specific damage to both the cell wall and photosynthetic membranes.

The surface of the *P. luridum* cells showed the development of blebs over 10–50% of available surfaces. These blebs in TEM thin sections appear to be composed of the lipopolysaccharide



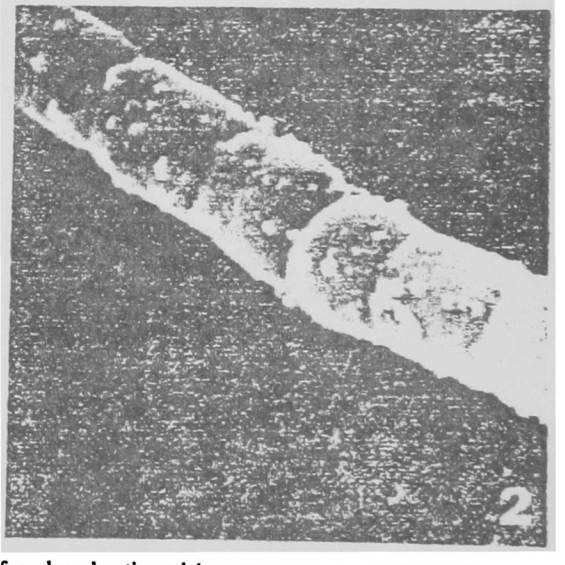


Fig. 1. Control cells of *P. luridum* grown in 1 part Difco algae broth and 1 part yeast extract (1.2%) for 24hr. The cell surface shows no significant protrusion or blebs. ×8300.

Fig. 2. Cells of a *P. luridum* trichome exposed to *Bdellovibrio bacteriovorus* culture supernatant for 12hr. The cyano-bacterial cell surface shows numerous blebs of variable sizes. ×17,000.

layer of the cyanobacterial cell wall. Incubation of the cyanobacteria in a bdellovibrio supernatant for over 24hr caused the trichomes to break into single cells showing various stages of lysis. This paralleled the degradation of trichomes heated to 55°C.

This photorespiratory phenomenon, because it is triggered by a relatively small toxic product of the bdellovibrios and because it results in the destruction of the cyanobacterial cell, may be a useful key to the better control of unwanted aqueous populations of these cyanobacteria.

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I 20 Entrapment and Lysis of Phormidium luridum by Colonial Spherules of a Myxococcus species.

J. C. BURNHAM*, S. A. COLLART, B. HIGHISON, Medical College of Ohio, Toledo, Ohio.

An isolate from a farm drainage ditch, Myxococcus PC02, is capable of rapidly lysing both agar and liquid cultures of the cyanobacterium, Phormidium luridum var. olivacea. P. luridum ($2x10^7$ cells/ml) was effected by parasite to host ratios of 1/10, 1/100, and 1/1000. Higher dilutions than 1:1 of Myxococcus cell-free supernatant were inactive in producing any effect on the P. luridum, suggesting only a slight extracellular enzymatic lytic activity. Long term transfer experiments show this gliding, microcyst-forming bacterium to be effective in maintaining reduced populations of cyanobacteria in aqueous autotrophic environments. Microscopy of the host-parasite interaction shows that lysis of the cyanobacterium occurs within spherules formed by the Myxococcus cells. Initially microclumps of several bacteria and cyanobacterial filaments form. These proceed to mature until a spherule diameter of 2 to 6 mm is achieved. The center of these densely green cyst-like spheres show cyanobacteria in various steps of degradation while the border is almost exclusively a tightly woven mass of the myxococci. Electron microscopy shows the formation of the clump to be aided by long (up to 3 um) lipopolysaccharide extrusions from the wall of the Myxococcus PC02 which appear to efficiently entangle the cyanobacterial filaments.

I 6 Myxobacterial Antagonism for Phorridium luridum
J. C. BURNHAM*, S. A. COLLART and B. W. HIGHISON
Medical College of Ohio, Toledo, Ohio 43699

Two strains of Myxococcus sp. (PCO2 and BGO2) isolated in Northwest Ohio show a unique ability to lyse aqueous populations of P. luridum. Both isolates form colonial clumps and spherules in either an axenic culture with a tryptone-salts medium or in a mixed culture with cyanobacteria with an autotrophic salts medium. When grown in the presence of P. luridum these myxococci envelope large numbers of cyanobacterial filaments a process which ends in the lysis of the cyanobacterial cells. In strain BGO2 the surface of the spherule can serve as a base for fruiting body formation. Although the PCO2 and BGO2 strains appear taxonomically similar to M. xanthus, the ATCC strain 25232 did not form lytic spherules or clumps. The Myxococcus BGO2 strain is able to lyse P. luridum populations averaging 5×10^6 cells/ml at cell ratios of at least per 50 P. luridum. The myxococci are able to present significant cyanobacterial regrowth in these mixed cultures for over 14 days. Maintenance of the mixed culture environment at pHs 6.0, 7.0, 8.0 and 9.0 did not alter their ability to control the P. luridum cultures. Serial 5 percent transfers every 7 days have demonstrated the long-term ability to survive on degraded cyanobacteria as well as the ability to continue antagonistic activity against viable P. luridum.